Towards understanding the effects of putative endocrine disruptors in the great pond snail Lymnaea stagnalis: Experimental and toxicokinetic-toxidcdynamic modelling approaches

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Henri Matisse, *The Snail*, 1953, gouache on paper cut-outs.
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Alpar Barsi

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VRIJE UNIVERSITEIT

Towards understanding the effects of putative endocrine disruptors in the great pond snail *Lymnaea stagnalis*: Experimental and toxicokinetic-toxicodynamic modelling approaches

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op gezag van de rector magnificus
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Alpar Barsi

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	dr. V. Ducrot

THESE / AGROCAMPUS OUEST

Sous le label de l'Université Européenne de Bretagne

pour obtenir le diplôme de :

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Alpar BARSI

Couplage d'approches expérimentales et modélisatrices pour l'étude des mécanismes d'effet de perturbateurs endocriniens chez la limnée des étangs *Lymnaea stagnalis*

soutenue le 14 janvier 2015 devant la commission d'Examen

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1. General introduction

Products that are used in daily life, such as plastic bottles, food, detergents, or cosmetics, contain numerous chemicals that may interfere with the endocrine system. The endocrine system plays a crucial role in maintaining homeostasis of the organism by integrating and coordinating metabolic and physiological processes relevant for development, growth, and reproduction [1]. Interactions of chemicals with the endocrine system may therefore negatively affect many aspects of human and animal health. The designation "endocrine disruptor" (ED) is given to an exogenous substance that alters the function(s) of the endocrine system, and consequently causes adverse effects in an intact organism, or its progeny, with consequences for population stability or recruitment[2, 3]. Plant protection products and biocides may unintentionally cause endocrine disruption in non-target animals. For instance, in aquatic molluscs, a concentration of only a few ng/L of some of these chemicals is enough to induce endocrine disruption and to consequently cause population declines [4]. To ensure a satisfactory protection level for wildlife, there is a need for improving the environmental risk assessment (ERA) of EDs.

To estimate the risk that chemicals may pose to wildlife, it is necessary to obtain information from toxicity tests about chemical effects on animals. Currently, the Organisation for Economic Co-operation and Development (OECD) is aiming at standardisation of test guidelines with molluscs [5]. Molluscs have been chosen because they are highly sensitive to chemical pollution and are of ecological and economical importance. Among the candidate species for the OECD standardisation is the freshwater gastropod *Lymnaea stagnalis*, the great pond snail. Compared to the majority of mollusc species, its endocrinology is better understood [6]. More importantly, the proposed OECD toxicity test protocol with the great pond snail advocates the use of "easy-to-test" reproduction and developmental endpoints (*e.g.*, fecundity and embryonic development), which may be associated with endocrine disruption ([5] and references therein). The proposed OECD toxicity test with the great pond snail provides standard summary statistics that is used for ERA, such as the no-observed effect concentration (NOEC, the highest tested concentration at which no statistically significant effects is observed relative to control) or the effect concentration (EC_x, the concentration at which a defined percentage of the effect is elicited relative to control). However, the summary statistics derived from standardised toxicity tests have been heavily criticised for lacking biological and ecological relevance [7-9].

The limitations of descriptive statistics can be overcome with the use of mechanistic effect models (MEMs). MEMs are considered promising tool for ERA because they can incorporate more ecology in ERA by addressing ecological complexity at different levels of biological organisation [10]. One type of MEMs are toxicokinetic-toxicodynamic (TKTD) models. These models simulate the processes that lead to toxicity in an individual organism over time [11, 12]. Thus, TKTD models can be used as a basis for linking toxic effects from the individual to the population level (see *e.g.*, [13]). As such, TKTD models can aid the use and interpretation of toxicity tests data and make ERA more robust [14]. Nevertheless, in comparison with standard statistical approaches in ecotoxicology, TKTD modelling is often considered data-hungry. Meeting modelling requirements may increase experimental efforts and costs, which, in conjunction with the relative complexity of TKTD models and the lack of modelling guidance, makes them less attractive for the use in risk assessments [15].

In this thesis, I address questions regarding the optimal design of toxicity tests for both standard ecotoxicological and TKTD modelling assessments. Furthermore, I explore the performance of a TKTD model for the interpretation of effects of putative EDs in the great pond snail. The ultimate aim of this work is to contribute to a better understanding of endocrine disruption in snails and to the improvements of the ERA of EDs.

1.1. Regulatory framework for endocrine disruptors

1.1.1. Concerns and testing of endocrine disruptors

Within the European Union (EU), marketing and use of chemical compounds is regulated on the basis that they should not generate endocrine disruption effects in humans and wildlife [16-19]. Regulations on plant protection products [17] and biocides [18] have established a hazard-based cut-off criterion for endocrine disruption. This means that potential endocrine disrupting chemicals might not get market approval, even though they can be used safely at low exposure levels [20].This precautionary measure was mainly based upon the assumption that there is no threshold for adversity of EDs [21]. This assumption comes primarily from epidemiological studies on carcinogens in humans, *e.g.* the development of breast cancer in women caused by an increased level of endogenous oestrogen hormones. The logic is as follows: because of pre-existing internal exposure to oestrogens, each molecule of externally added oestrogenic agent adds to the internal load, thereby exhibiting activity in a threshold-independent manner leading to adversity [1]. The problem with this concept is that the existence of a threshold cannot be proven nor rejected because all methods for measuring effects have their limits of detection, obscuring thresholds even if they exist [1]. Whether or not a threshold for adverse effects of EDs really exists, and its relevance for ecotoxicology, is still unclear.

Based on empirical evidence, other properties of EDs raise concerns for wildlife. First, some EDs exert adverse effects at low concentrations, *i.e.* at concentrations below the range typically used in toxicological studies [22]. A well-known example of the effects of EDs is the biocide tributyltin (TBT) in aquatic gastropods [4]. Many field and laboratory experiments have proven the ability of TBT to induce imposex, *i.e.* penis development in female gonochoristic snails, or intersex, *i.e.* oogenesis is supplanted by spermatogenesis, at very low TBT concentrations (a few ng/L). These abnormalities prevented normal reproduction of snails, devastating their populations. Furthermore, some EDs may elicit non-monotonic concentration responses (*e.g.*, U-shaped or inverted U-shaped response curves, Figure 1.1) in both vertebrates and invertebrates [23]. This opens up the possibility to miss the effects from standard toxicity tests if a small range of concentrations is tested. In regulatory ecotoxicology, an S-shape concentration response is generally assumed. Therefore, if the effects that are observed at higher exposure concentrations are extrapolated to lower ones, potential effects at low concentration levels could be missed, which may lead to inappropriate risk assessments [24]. Finally, some EDs may induce delayed toxicity (*e.g.* exposures to some dioxins in perinatal life have a negative effect on semen quality in adulthood), or be effective (or have the most prominent effects) at different life stages [25].

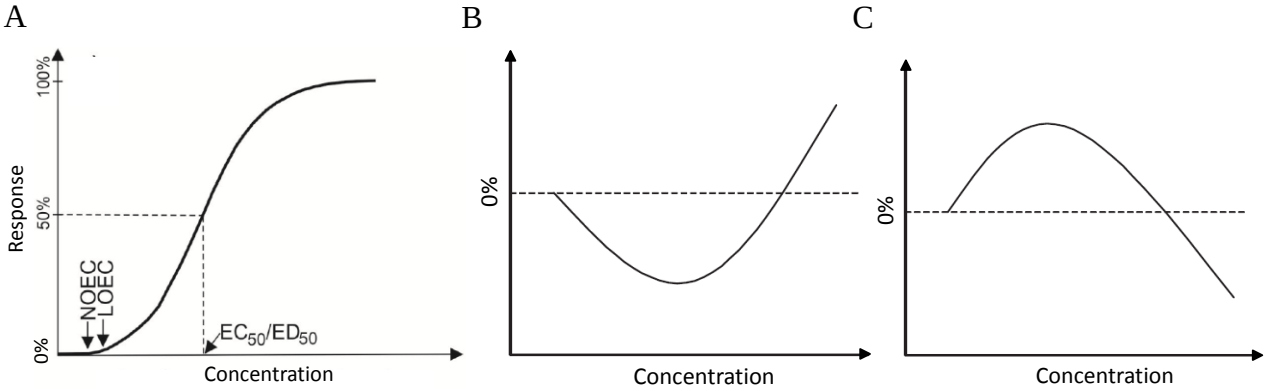

Figure 1.1. Different types of response to toxicant exposure: A) classical (sigmoid) concentration-response curve (greater effect with increasing dose). NOEC, no-observed effect concentration; LOEC, lowest-observed effect concentration; EC, effective concentration; ED, effective dose. B) U-shaped concentration-response curve (low-concentration inhibitory and high-concentration stimulatory response), and C) inverted-U-shaped concentration-response curve (low-concentration stimulatory and high-concentration inhibitory response). Adapted from [26, 27].

Despite the existing regulations on marketing and use of chemicals with endocrine disrupting properties, the scientific criteria on how to identify and assess adverse effects of EDs on wildlife have not been provided yet. Interim criteria have been proposed, in which a proper demonstration of endocrine disruption requires establishing causal links between endocrine activity (as assessed *in vitro*) and adverse effects (as assessed *in vivo*) on an intact organism or a (sub)population [20, 28]. To aid identification and assessment of EDs, the OECD has developed the Conceptual Framework for Testing and Assessment of Endocrine Disrupters (EDTA, Figure 1.2). The EDTA framework guides researchers to appropriate data sources and OECD standardised test methods (already available, or those under development) for the assessment of endocrine active chemicals (EACs) and EDs. The EDTA framework covers "only" the modalities of endocrine disruption for which sufficient knowledge exists. Those are oestrogen, androgen, thyroid, and steroidogenesis modalities. This knowledge mostly comes from vertebrate studies. Other pathways for endocrine disruption, such as retinoid X or peroxisome proliferator-activated receptor signaling pathways, and many others, have not been covered yet [28].

It can be seen from the EDTA framework that there is a solid basis for the evaluation of chemicals on endocrine activity and adversity in vertebrates, particularly mammals and fish. A large amount of useful information about the chemical activity and the effects can be obtained from the literature, existing mechanistic *in vitro* and *in vivo* toxicity tests (*i.e.*, tests providing information on the mechanism of action of chemicals, levels 2 and 3 of the EDTA framework) and apical whole-organism toxicity tests (levels 4 and 5 of the EDTA framework). However, it is also clear from Figure 1.2 that mechanistic tests for invertebrates are missing (levels 2 and 3). Hence, for this group of organisms there is no means to demonstrate endocrine activity of chemicals, and thereby no means to establish the adversity due to endocrine disruption. Indeed, adverse effects in invertebrates can be assessed from partial or full life-cycle tests, as defined for levels 4 and 5 of the EDTA framework, but no information about the mechanism of the effects can be obtained from the apical tests. The hazard-based cut-off criterion for EDs is thus currently not applicable to invertebrates; as a consequence, chemicals will go through the standard risk assessment procedures until a complete suite of specific tests for endocrine disruption is available.

1.1.2. Development of the OECD standardised toxicity tests with molluscs

Molluscs are the second largest animal phylum in terms of the number of species. Molluscs cover a variety of terrestrial and aquatic habitats. Within both vertebrate and invertebrate groups, molluscs show the greatest sensitivity towards some chemicals, including EDs. Many features of the endocrine system are shared between molluscs and vertebrates, such as the presence of steroid hormones. Therefore, molluscs have been recognized as a relevant group for endocrine disruption testing for ERA purposes. This has initiated the development and validation of the OECD test guidelines on molluscs (apical tests at levels 4 and 5 of the EDTA framework, Figure 1.2), which will be sensitive to a wide range of chemicals and not strictly to EDs [5]. These tests are designed to provide test results that can be analysed using descriptive statistics (*e.g.*, NOEC, EC_x); they are not intended for TKTD analyses.

The detailed review paper on existing test protocols [5] focuses on three relevant species for the development of standard toxicity tests with molluscs: the aquatic gastropods *Potamopyrgus antipodarum* and *L. stagnalis*, and the bivalve *Crassostrea gigas*. The criteria for choosing these species among other candidates are based upon the current knowledge about their physiology, and the feasibility of rearing animals under laboratory conditions. The proposed species represent different reproductive strategies, *i.e. P. antipodarum* is a parthenogenetic gonochorist while *L. stagnalis* and *C. gigas* are hermaphrodites (simultaneous and sequential hermaphrodites, respectively). These three species also represent the animals inhabiting different environments, *i.e. P. antipodarum*, *L. stagnalis*, and *C. gigas* are brackish, freshwater, and marine species, respectively.

	Mammalian and non-mammalian toxicology	
Level 1 Existing data and non-test information.	• Physical and chemical properties, e.g. MW reactivity, volatility, biodegradability • All available (eco)toxicological data from standardised or non-standardised tests • Read-across, chemical categories, QSARs and other *in silico* predictions, and ADME model predictions	
Level 2 *In vitro* assays providing data about selected endocrine mechanism(s) / pathways(s) (mammalian and non-mammalian methods).	• Oestrogen or androgen receptor binding affinity • Oestrogen receptor transactivation (OECD TG 455 – OECD TG 457) • Androgen or thyroid transactivation (if/when TGs are available) • Steroidogenesis in vitro (OECD TG 456) • MCF-7 cell proliferation assays (ER ant/agonist) • Other assays as appropriate	
	Mammalian toxicology	**Non-mammalian toxicology**
Level 3 *In vivo* assays providing data about selected endocrine mechanism(s) / pathway(s).	• Uterotrophic assay (OECD TG 440) • Hershberger assay (OECD TG 441)	• *Xenopus* embryo thyroid signalling assay (When/if TG is available) • Amphibian metamorphosis assay (OECD TG 231) • Fish reproductive screening assay (OECD TG 229) • Fish screening assay (OECD TG 230) • Androgenised female stickleback screen (GD 140)
Level 4 *In vivo* assays providing data on adverse effects on endocrine relevant endpoints.	• Repeated dose 28-day study (OECD TG 407) • Repeated dose 90-day study (OECD TG 408) • 1-generation reproduction toxicity study (OECD TG 415) • Male pubertal assay (see GD 150, Chapter C4.3) • Female pubertal assay (see GD 150, Chapter C4.4) • Intact adult male endocrine screening assay (see GD 150, Chapter Annex 2.5) • Prenatal developmental toxicity study (OECD TG 414) • Chronic toxicity and carcinogenicity studies (OECD TG 451-3) • Reproductive screening test (OECD TG 421 if enhanced) • Combined 28-day/reproductive screening assay (OECD TG 422 if enhanced) • Developmental neurotoxicity (OECD TG 426)	• Fish sexual development test (OECD TG 234) • Fish reproduction partial life-cycle (PLC) test (when/if TG is Available) • Larval amphibian growth and development assay (when TG is available) • Avian reproduction assay (OECD TG 206) • Mollusc PLC assays (when TG is available) • Chironomid toxicity test (TG 218-219) • *Daphnia* reproduction test (with male induction) (OECD TG 211) • Earthworm reproduction test (OECD TG 222) • *Enchytraeidae* reproduction test (OECD TG 220) • Sediment-water *Lumbriculus* toxicity test using spiked sediment (OECD TG 225) • Predatory mite reproduction test in soil (OECD TG 226) • Collembolan reproduction test in soil (TG OECD 232)
Level 5 *In vivo* assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism.	• Extended one-generation reproductive toxicity study (OECD TG 443) • 2-Generation reproduction toxicity study (OECD TG 416 most recent update)	• Fish full life-cycle (FLC) toxicity test (when TG is available) • Medaka multigeneration test (when TG is available) • Avian 2 generation reproductive toxicity assay (when TG is available) • Mysid FLC toxicity test (when TG is available) • Copepod reproduction and development test (when TG is available) • Sediment-water chironomid FLC toxicity test (OECD TG 233) • Mollusc FLC assays (when TG is available) • *Daphnia* multigeneration assay (if TG is available)

Figure 1.2. The OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupters (quoted from [29]).

P. antipodarum has been considered more suitable for the development of a partial life-cycle test, while the other two species are considered for development of full life-cycle tests [5]. Practically, toxicity tests will be validated first as partial life-cycle tests, and for *P. antipodarum* and *L. stagnalis* only. The process of the OECD standardisation is progressing, and currently partial life-cycle tests with *P. antipodarum* and *L. stagnalis* have been "pre-validated" (a ring-test has shown a satisfactory reproducibility of results between different laboratories).

1.2. Dynamic energy budget theory and its application in ecotoxicological research

In ecotoxicology and the ERA of chemicals, common quantifiers of toxic effects are the NOEC or EC_x. As mentioned before, the application of the NOEC and EC_x concepts have been widely criticised [7-9], and the limitations of these concepts are summarised in *e.g.* Jager *et al.* [12]. For example, both quantifiers are endpoint specific and depend on exposure duration. The NOEC may be associated with substantial effects (*e.g.*, 10-34%), which relates to the general limitations of statistical testing [30]. Although the EC_x and the confidence intervals can be estimated under certain assumptions, the model behind is purely descriptive and biologically meaningless (which holds for the NOEC as well). Overall, these quantifiers, based on descriptive statistics, do not increase our understanding of the mechanisms of toxic effects of chemicals and hamper the interpretation of the effects observed in toxicity tests.

Applying classical methodology for toxicity assessments for EDs may prevent the detection of biologically significant effects. Particular concern in this context are low-concentration responses. Furthermore, the non-monotonic concentration responses that are regularly reported for EDs cannot be properly analysed with classic statistical approaches. Moreover, possible transient effects of EDs can be missed as the assessments are made only for a single time point (end of a test).

By using TKTD modelling approaches the limitations of descriptive statistics may be overcome. TKTD models that can be used for the assessments of chemicals, including EDs, are *e.g.* those based on Dynamic Energy Budget (DEB) theory.

DEB theory explains feeding, development, growth, reproduction, and aging of an individual organism over time within one coherent framework ([31, 32]). The theory sets the rules for energy acquisition and allocation to certain metabolic and physiological processes that define the life-cycle traits of an individual. Using DEB theory to study effects of EDs is relevant as long as the effects have a clear energetic component (*e.g.*, effects on size or reproductive output); in contrast, the application of DEB theory is less likely to be useful for endpoints such as changes in behaviour or sex ratios *etc*. Energy allocation between the soma and maturation/reproduction is specified by the "kappa rule": a fixed fraction of energy κ is allocated to growth and somatic maintenance (the latter costs have to be paid first), while the remaining 1-κ fraction is used for maturation, reproduction and maturity maintenance (Figure 1.3). The value of κ is assumed to be under hormonal control, and is generally constant over the life cycle. Some environmental factors such as duration of daylight or parasitic infections may influence the value of κ in *L. stagnalis*, which have dramatic consequences for life history [31, 33, 34].

One of the major advantages of using DEB models in ecotoxicology is that they account for the all available information about chemical effects on multiple endpoints over time [35]. In this way, DEB models provide a robust, biologically and ecologically meaningful, and theoretically consistent explanation of toxic effects. Furthermore, DEB models can serve as a good basis for extrapolations to study the population-level consequences of toxic effects (see *e.g.*, [36]). DEB theory can also help extrapolations of toxic effects among species and chemicals [37]. Examples of applications of DEB models in ecotoxicology is available at www.debtox.info/papers_debtox.php while a review of their applications in population ecotoxicology can be found in Jager *et al.* [38].

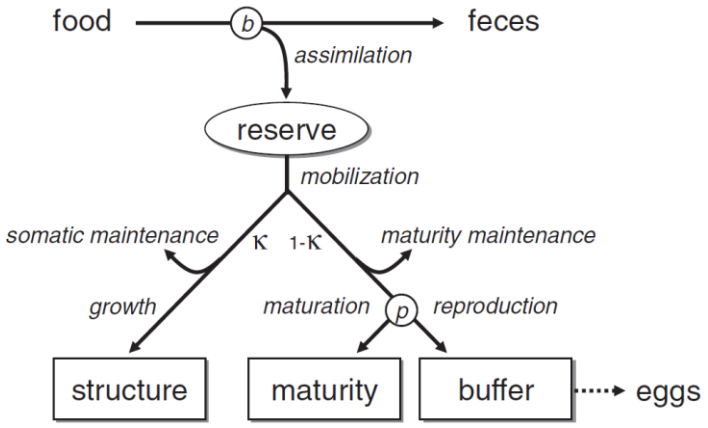

Figure 1.3. Scheme of the standard DEB animal model. The two nodes represent the switches in the life-cycle trait at birth (*b*, start of feeding) and puberty (*p*, switch investment from maturation to production of gametes) (quoted from [39]).

The added value of DEB models for analysing toxicity tests data has been recognised by regulatory bodies, and one simplified DEB model has been included into guidance for ecotoxicological assessments [40, 41]. Despite the promising features of DEB theory in ecotoxicology, it is rarely applied for regulatory purposes. DEB and many other MEMs are relatively complex, they are based on explicit simplifying assumptions of biology, and for their calibration and parameterisation data from non-standardised tests are in many cases required. Furthermore, there is a lack of guidance on when and how to apply ecological models in general [15]. Initiatives to address some of these questions have recently been made, *e.g.* EFSA's (the European Food Safety Authority) opinion on good modelling practice [42] or the MODELINK workshop, which gathered people from regulatory bodies, academia, and industry [15]. Furthermore, the issues are also tackled in the most recent guidance documents on the risk assessment of plant protection products for birds and mammals [43], for bees [44], and for aquatic organisms in edge-of-field surface waters [45]. These guidance documents provide information on a possibility of using MEMs in the current ERA scheme, but do not recommend any particular model: the choice of the most appropriate model is left to the modeller.

According to DEB theory, the internal concentration of a chemical exerts the effects by changing the value of a target (metabolic) parameter after a threshold has been reached. This change in the model parameter value subsequently affects the individual life-history traits in a specific manner (*e.g.*, growth and reproduction). The change of the parameter's value is taken as a linear function of the internal concentration above the threshold. However, this does not imply that the individual trait is changing linearly (the relationship between a parameter's value and the traits is not necessarily linear). Any model parameter may be affected by a toxicant, and possibly even several at the same time. The parameter (or set of parameters) that is affected is called the metabolic mode of action (mMoA) of a chemical, and each mMoA has specific consequences for the life-history traits over time [46]. Some of the commonly reported mMoAs are the surface-specific assimilation rate and the costs for structural growth (Figure 1.3).

In most DEB applications in ecotoxicology, the same set of generic mMoAs is used to model adverse effects of all chemicals in all species [46, 47]. It is unclear whether DEB models with this set of mMoAs are able to describe patterns of the effects of EDs on life-cycle traits without additional physiological assumptions. It is possible that the effects of EDs can readily be described by the current models and mMoAs because the endocrine system controls the metabolic processes that underlie the mMoAs of toxicants. For example, in the marine polychaete *Capitella telata*, effects of the oestrogenic chemical nonylphenol were well described without additional hypotheses about the animal's physiology or the chemical's mode of action [39]. As EDs affect processes relevant for energy flow in the organism, it would be interesting to check whether the model

parameter κ is affected by EDs in *L. stagnalis*. Furthermore, if non-monotonic concentration responses are observed, it is unlikely that they can be modelled by using a linear-with-threshold relationship between the internal concentration and the parameter value, and mechanism-based model modifications will be needed.

A range of practical models has been derived from DEB theory, but most of the ecotoxicological applications rely on a simplification often referred as DEBtox [48-50]. Because it contains fewer details about the animal's physiology than the standard DEB animal model [32], it is easier to parameterise and its calibration is less data demanding. It thus better suits the available data from toxicity tests. In this PhD thesis I apply a more recent simplification, called DEBkiss [51]. In contrast to DEBtox, this model sports an explicit mass balance, which allows, for example, the implementation of starvation rules that respect mass and energy conservation. Furthermore, DEBkiss does not divide biomass into a reserve and structure component, and explicitly includes the embryonic stage. These model features make DEBkiss relatively easily applicable in the analysis of ecotoxicity tests data, particularly of invertebrates. The DEBkiss model has shown to provide a good description of the life cycle of *L. stagnalis* [51].

1.3. The great pond snail

The test species used in my work is the freshwater gastropod *L. stagnalis*, the great pond snail (Figure 1.4). It is a pulmonate species that belongs to the order *Basommatophora*, family *Lymnaeidae*. *L. stagnalis* is a Holarctic species that inhabits stagnant or slow-running waters. Its diet constitutes organic plant and animal material. Depending of the environmental conditions, the snail reaches a size (shell length) between 20 and 60 mm [5]. *L. stagnalis* is a simultaneous hermaphroditic species that sports out-crossing, but in the absence of conspecifics self-fertilisation may occur. In laboratory conditions *L. stagnalis* becomes sexually mature and starts laying eggs at approximately 21-24 mm [33]. Absence of a mate may prolong the time to first oviposition [52]. Clutches that contain 50 to 150 eggs are laid on the substrate, usually plants. The number of eggs in a clutch is positively correlated with the snail size, but can vary depending on the presence of a sexual mate [53].

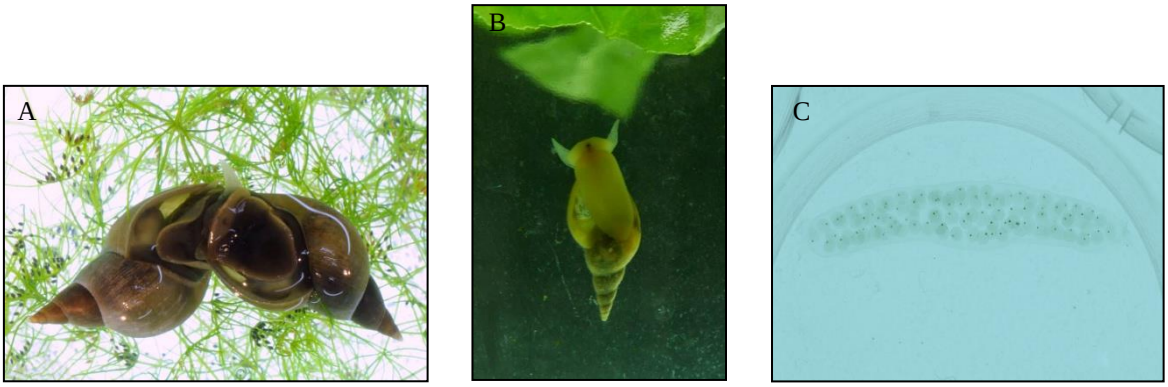

Figure 1.4. *L. stagnalis*, the great pond snail; A) snails crawling on each other, B) an individual, and C) an egg clutch.

1.3.1. Neuroendocrine control and steroids in L. stagnalis

In contrast to the majority of mollusc species, the functioning of the neuroendocrine system of *L. stagnalis* is relatively well understood, as reviewed in [6, 54]. Growth and reproduction are under the interdependent control of various types of neurons and endocrine cells. These cells communicate by secretion of various types of proteins and peptides to achieve neuroendocrine control. For example, light green cells of the cerebral ganglia control body growth, shell formation, protein and glycogen metabolism, while the control of reproduction is achieved by caudo-dorsal cells and dorsal bodies situated also in the cerebral ganglia. Proteins and peptides that are partially

involved in the control of reproduction are shown in Figure 1.5, while the neuroendocrine control of reproduction is depicted in Figure 1.6.

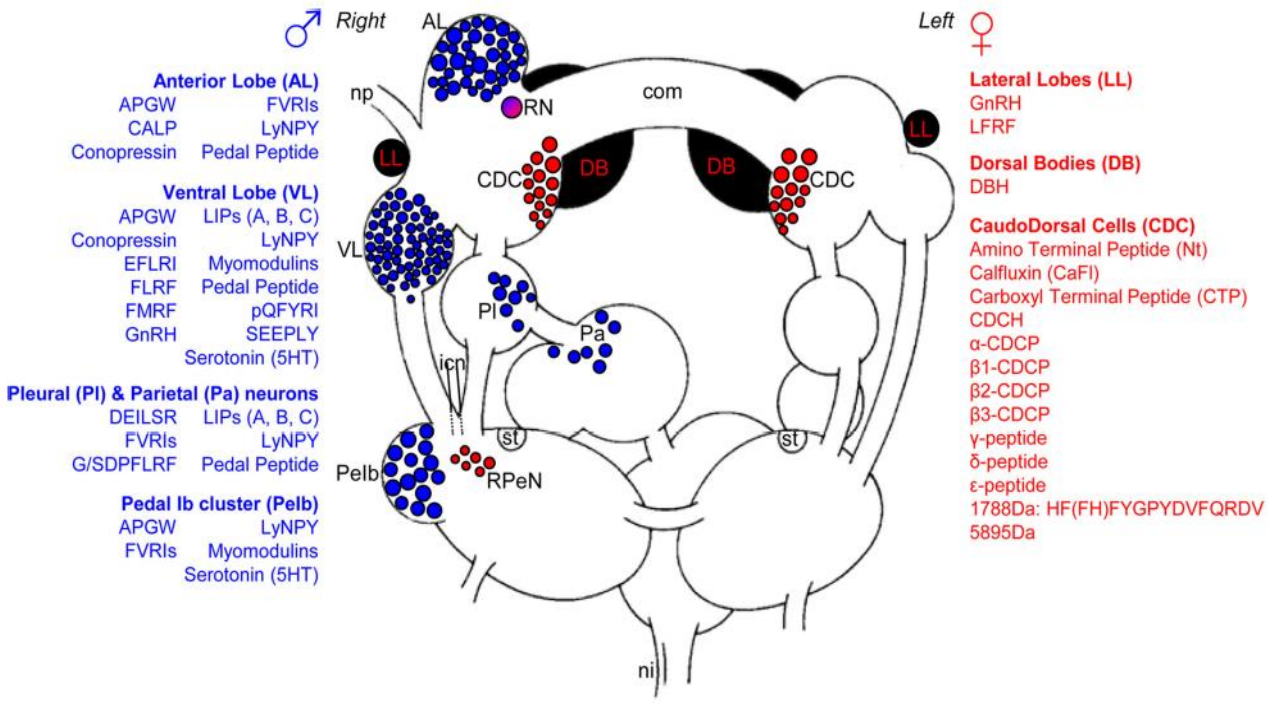

Figure 1.5. Schematic drawing of the involved ganglia of the central nervous system. The blue and red areas show, respectively, the neuronal clusters that are involved in male and female reproduction. The substances involved in male reproduction are also indicated in blue; the female ones are listed in red. com, cerebral commissure; icn, inferior cervical nerve; ni, nervus intestinalis; np, nervus penis; RN, ring neuron; RPeN, right pedal neurons; st statocyst (quoted from [54] and [55]).

Effects of exposure to vertebrate steroid hormones and their mimics in gastropods were studied mostly in prosobranch snails. These chemicals exert oestrogenic and androgenic effects similar to those observed in vertebrates. So far, the great pond snail has not been tested extensively on these chemicals. Only few studies are available on the effects of the oestrogenic chemicals 17α-ethynyloestradiol, nonylphenol, and chlordecone [56-59], and androgenic chemicals testosterone, 17α-methyltestosterone, and tributyltin [57, 59, 60]. Some anti-androgenic chemicals also have been tested, like vinclozolin, fenitrothion, and cyproterone acetate [61].

Although exposure to steroids may induce adverse effects in some molluscs, it is not clear whether they are naturally present in the molluscan organism and if so, what their exact role is [62, 63]. According to Lafont and Mathieu [64], vertebrate-type steroid hormones are naturally present in gastropods. However, the steroid biosynthesis pathways have not been identified in molluscs yet. The existence of steroid hormones in snails may just be a result from bioaccumulation of these hormones from the environment [62]. Giusti *et al.* [59] have demonstrated the capacity of *L. stagnalis* to accumulate testosterone from its surrounding, but also argued for the possibility that testosterone in this snail species can be synthesised *de novo*.

Steroid hormones play crucial roles in the neuroendocrine control of development, growth and reproduction of vertebrates [65]. In some molluscs, steroids induce responses, as demonstrated in the examples of imposex and intersex in prosobranchs, but the mechanisms behind these effects still remain unknown. Steroids may also have a non-endocrine-related role in *e.g.* chemical communication, defence, and digestive physiology of molluscs [64].

Overall, the role of steroid hormones in the neuroendocrine regulation of reproduction in molluscs is still poorly understood. Some of the unanswered questions regarding molluscs and steroids are: are steroids synthesised *de novo*; do they really have a hormonal role in reproduction

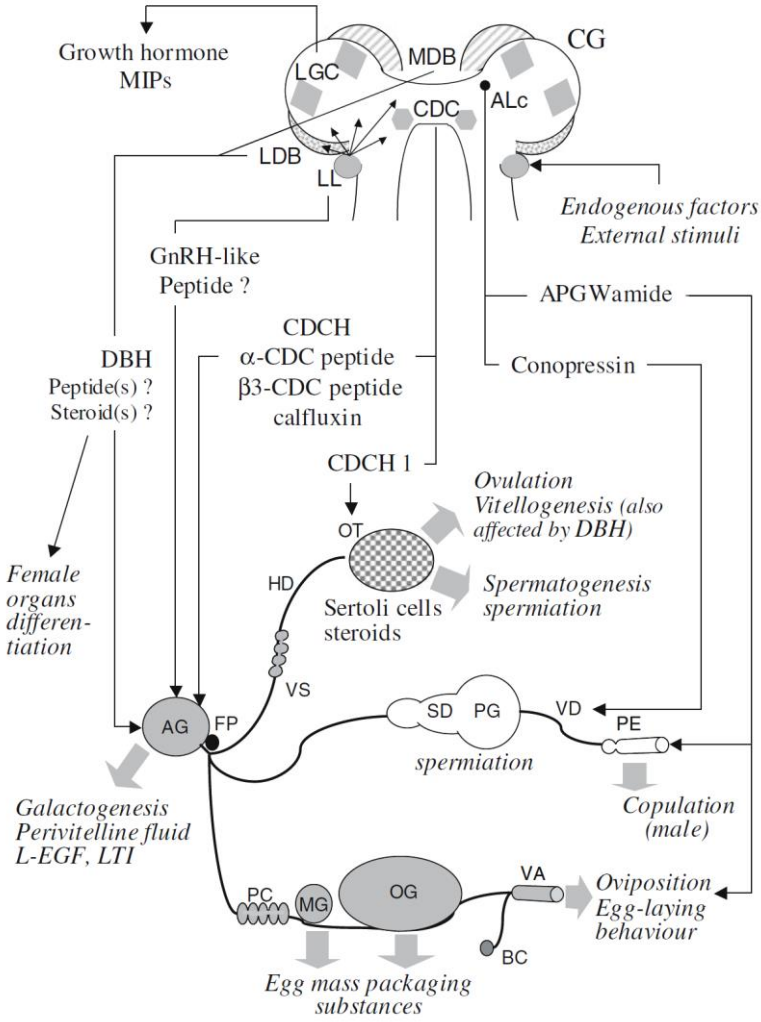

Figure 1.6. A schematic view of neuroendocrine control of reproduction in Basommatophora. AG, albumen gland; ALc, cells of the cerebral ganglia anterior lobes; APGW, Ala-Pro-Gly- Trp-NH2 tetrapeptide; BC, bursa copulatrix; CDC, caudo-dorsal cells; CDCH, caudo-dorsal cell hormone; CG, cerebral ganglia; DBH, dorsal body hormone; FP, fertilization pocket; GnRH, gonadotropin-releasing hormone; HD, hermaphrodite duct; LDB, lateral dorsal bodies; L-EGF, Lymnaea epidermal growth factor; LGC, light green cells; LL, lateral lobes; LTI, Lymnaea trypsin inhibitor; MDB, median dorsal bodies; MG, muciparous gland; MIP, molluscan insulin-like peptide; OG, oothecal gland; OT, ovotestis; PC, pars contorta; PE, penis; PG, prostate gland; SD, sperm duct; VA, vagina; VD, vas deferens; VS, vesiculae seminales (quoted from [6]).

of snails, and if yes, what is that role; what are the biosynthetic pathways of steroids; are steroid receptors present in *L. stagnalis*; if yes, do they interfere with reproduction, or do they have completely different roles? These are only some of pending issues that have to be solved in the future to allow for a proper risk assessment of suspected EDs in aquatic snails.

1.4. Thesis overview

In this PhD thesis, I investigated the sensitivity of *L. stagnalis* to vertebrate-like steroid hormones and their mimics, using several chemicals that have already been studied and some that have not. Particularly, the focus was to see whether these chemicals induce effects at low concentration ranges, whether they induce non-monotonic concentration responses, and whether there are certain windows of sensitivity of snails to EDs. Besides commonly observed endpoints such as cumulative fecundity and oviposition, I examined other possibly affected life-cycle traits, *e.g.* growth and development. To answer some of the research questions, I conducted laboratory toxicity tests,

which should be sensitive enough to detect adverse effects of endocrine-disrupting chemicals. Part of my project was dedicated to modelling the chemical effects using a DEB model. This required optimisation of the currently proposed OECD test protocols [5] to obtain data suitable for TKTD modelling. A key question in this respect was whether explaining the effects of EDs in *L. stagnalis* requires additional assumptions about the physiology of the snails and/or the mMoA of the chemicals that are not included in existing DEB models. I will discuss to which extent understanding the effect of EDs on the energy budget of *L. stagnalis* may complement our knowledge about endocrine disruption, and how this knowledge can advance ERA of EDs.

The issues are addressed in the following four chapters:

Chapter two explores the impacts of two androgenic chemicals, tributyltin (TBT) and triphenyltin (TPT), on reproduction of *L. stagnalis*. Based upon the observations on prosobranch snails, it is expected that the two chemicals affect sub-lethal endpoints in the great pond snail (*e.g.*, reproduction). As the chemicals are considered to be EDs in prosobranchs, we may observe low-concentration effects or non-monotonic concentration responses. This was tested in 21-day toxicity tests, designed upon the proposed OECD protocol with *L. stagnalis* [5]. Effects were analysed with classic ecotoxicological approaches, *i.e.* hypothesis testing and statistical modelling, yielding the standard toxicity quantifiers: NOEC and EC_x.

Chapter three discusses the practical and theoretical considerations for test design to accommodate TKTD models in ecotoxicology. TKTD modelling places different demands on test design compared to classical ecotoxicological analyses. Guidance on the most appropriate test design to calibrate TKTD models is lacking, which may be part of the reason why ecotoxicologists and risk assessors are reluctant to use (results from) these models. In a case study with acetone and the great pond snail, we evaluated the benefits of using different types of data for a DEB-based TKTD model calibration. We explored how standard toxicity test data, as well as data on additional endpoints, affect model calibration. The optimal data set for calibration of the TKTD model DEBkiss is discussed in relation to the data already available from standard tests to scrutinize the costs and benefits of additional experimental effort. Finally, with the model calibrated on test data for sub-adult and adult snails, we tested its ability to predict the effects of acetone on embryonic development.

Chapter four demonstrates the applicability of the DEBkiss model to assess adverse effects of TBT in *L. stagnalis* in an integrative way. We tested whether the effects of this putative ED can be described with metabolic modes of action that are already implemented in DEB models, or whether new assumptions about toxicity are needed. For this purpose, we parameterised and calibrated the DEBkiss model based upon data obtained from two independent toxicity tests on sub-adult and adult snails, simultaneously. We discuss the links between sub-cellular and metabolic targets of TBT in the organism.

Chapter five investigates effects of oestrogenic chemicals, *i.e.* 17α-ethynyloestradiol and alkylphenols on survival, growth, development, and reproduction of snails. Based upon findings in other studies on prosobranch snails, it is expected that the oestrogenic chemicals would stimulate reproduction of *L. stagnalis*. To test this hypothesis we performed a number of toxicity tests with *L. stagnalis*, which differed in their design. The results are discussed in the light of the appropriateness of the tests to detect possibly endocrine related effects. Furthermore, the adequacy of 17α-ethynyloestradiol as positive control for reproductive effects in toxicity tests with *L. stagnalis* is discussed.

Chapter six presents a synthesis of the findings described in the previous chapters and provides recommendations for future researches.

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2. Reproductive impacts of tributyltin (TBT) and triphenyltin (TPT) in the hermaphroditic freshwater gastropod *Lymnaea stagnalis*

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Abstract

Tributyltin (TBT) and triphenyltin (TPT) are emblematic endocrine disruptors, which have been mostly studied in gonochoric prosobranchs. Although both compounds can simultaneously occur in the environment, they have mainly been tested separately for their effects on snail reproduction. Because large discrepancies in experimental conditions occurred in these tests, the present study aimed to compare the relative toxicity of TBT and TPT under similar laboratory conditions in the range of 0 ng Sn/L to 600 ng Sn/L. Tests were performed on the simultaneous hermaphrodite *Lymnaea stagnalis*, a freshwater snail in which effects of TPT were unknown. Survival, shell length, and reproduction were monitored in a 21-d semistatic test. Frequency of abnormal eggs was assessed as an additional endpoint. TPT hampered survival while TBT did not. Major effects on shell solidity and reproduction were observed for both compounds, reproductive outputs being more severely hampered by TBT than by TPT. Considering the frequency of abnormal eggs allowed increasing test sensitivity, because snail responses to TBT could be detected at concentrations as low as 19 ng Sn/L. However, the putative mode of action of the two compounds could not be deduced from the structure of the molecules or from the response of apical endpoints. Sensitivity *of L. stagnalis* to TBT and TPT was compared with the sensitivity of prosobranch molluscs with different habitats and different reproductive strategies.

2.1. Introduction

Organotins have been used for more than 50 years, mainly as pesticides, antifungal agents and, in the case of the widely used tributyltin (TBT) and triphenyltin (TPT), as the active biocides of antifouling paints [1]. Their use in antifouling paints was prohibited in 2008 [2], but residues can still be found both in marine environments and in freshwater ecosystems at concentrations up to, for example, 7.1 µg TBT/L [3]. Therefore, the assessment of toxic effects of these compounds to aquatic, freshwater wildlife is still relevant. To date, TBT and TPT have mostly been investigated separately as single compounds. These studies have pointed out some similarities between TPT and TBT regarding their physicochemical properties and biological effects. Indeed, these molecules have a closely related structure: a tetravalent tin core with either three butyls (TBT) or three phenyls (TPT). These compounds are fairly persistent in the environment, being retained in sediments [4-6]. Furthermore, they penetrate biological systems and can be stored at high concentrations in lipid-rich tissues of aquatic organisms; for example, TBT levels up to 233 ng/g have been recorded in retail mollusc products [7]. Both TBT and TPT accumulate preferentially in the hepatopancreas and kidneys and at lower levels in the heart and brain [8-10]. Consequently, both TBT and TPT can induce adverse effects on wildlife, which have been studied mainly in marine molluscs.

As early as 1975, adverse impacts of TBT— shell calcification in adults leading to stunted growth—were observed in the oyster *Crassostrea giga* [11]. At low concentrations (nanograms per liter), TBT was shown to induce the imposition of male sex organs in female prosobranchs [12]. This phenomenon, named *imposex*, has now been reported to occur in more than 200 mesogastropod and neogastropod marine and freshwater species [13]. At highly contaminated sites, females can be sterilised or even killed, which may affect population dynamics [14]. In addition, TPT was shown to induce reproductive failure through imposex in different gastropod species [15-17]. A number of studies reported other types of reproductive alterations in freshwater species due to exposure to organotins [18-24]. For instance, exposure of *Lymnaea stagnalis* to TBT at 1 µg Sn/L was shown to induce abnormal development of embryos (absence of shell), and a decrease in egg hatchability; exposure to 10 µg Sn/L led to a complete hatching failure [19].

Organotins generally occur in the environment as mixtures of TBT and TPT and their derivatives (*i.e.*, dibutyltin (DBT), monobutyltin (MBT), diphenyltin (DPT), and monophenyltin (MPT))[25-26]. Therefore, a growing number of studies are devoted to the study of the relative toxicity of these compounds, which constitutes the first step toward the study of mixture toxicity. Comparisons of bioaccumulation and biological responses to TBT and TPT have already been done in several gastropod species such as the muricids *Thais clavigera* and *Thais bronni* [27], as well as *Bolinus brandaris* and *Hexaplex trunculus* [28]. These studies allow a straightforward comparison of TBT and TPT effects under similar test designs and experimental conditions and using similar test endpoints. This ensures both quantitative and qualitative reliability of effect comparisons through the avoidance of experimental confounding factors, which often occur when data from different studies are compared. To date, these comparative studies have dealt only with gonochoric species, in which toxic effects can be assessed through direct monitoring of the morphological changes in the sexual apparatus. Furthermore, impacts on endocrinology and especially on sexual steroids are more easily understood in species that exhibit separated genders, in which the male and female hormonal reproductive pathways can be distinguished.

Effects on the reproductive pathways and performances in hermaphroditic species are more subtle. Probably due to the complex sexual apparatus and the variety of reproduction strategies in simultaneous hermaphrodite gastropods (*e.g.*, selfing vs. outcrossing) [29], the effects of organotins in these animals have not yet been extensively investigated [20]. In particular, it is important to investigate suitable endpoints that will allow the highlighting of reproductive effects in species in which sex differentiation cannot be used as an effect criterion. To date, most studies have been conducted using the great pond snail *L. stagnalis*. This holarctic freshwater snail lives in ponds and lakes. It is a simultaneous hermaphrodite, which can outcross and self-fertilise [30]. It has been

identified as one of the most relevant mollusc species for assessing reprotoxic effects of chemicals [20, 29, 31-33]. Because its neurohormonal control of reproduction is reasonably well understood compared with other mollusc species [32-35], and also because it has been shown to be sensitive to endocrine disruptors [19, 21, 24, 31, 35], standard Organisation for Economic Cooperation and Development (OECD) test guidelines for apical reprotoxicity tests (both partial and full life-cycle tests) with *L. stagnalis* are currently under development.

In the present study, we aimed to investigate the reproductive effects of the emblematic organotin compounds TBT and TPT, known as endocrine disruptors in some mollusc species, in the hermaphroditic freshwater snail *L. stagnalis*. The effects of TBT and TPT were studied comparatively under the same controlled laboratory conditions. Reproductive effects were assessed through a set of complementary apical endpoints (*i.e.*, number of egg clutches, number of eggs, and frequency and type of abnormal eggs). Adult survival and growth also were monitored. Effects of TBT and TPT were compared both qualitatively and quantitatively. The influence of the structure of the molecules on the biological responses and possible corresponding modes of toxic action are discussed. Moreover, biological responses of this hermaphroditic snail to TBT and TPT were compared with available data in other mollusc species.

2.2. Material and methods

2.2.1. Test organisms

Lymnaea stagnalis (Linnaeus, 1758; Mollusca, Gastropoda, Panpulmonata, Heterobranchia) were reared at the Experimental Unit of Aquatic Ecology and Ecotoxicology (National Institute for Agricultural Research, Rennes, France) under laboratory conditions as previously described [30]. The culture medium consisted of dechlorinated, charcoal-filtered tap water with the following physicochemical characteristics: pH 7.7 ± 0.2, conductivity 623 ± 60 µS/cm, dissolved oxygen 7.3 ± 2 mg/L, and water hardness 254 ± 7 mg $CaCO_3$/L. Rearing conditions were as follow: temperature 20 ± 1 °C, photoperiod 14:10-h light:dark, and light intensity 155 ± 35 lux. Snails (RENILYS® strain) were fed three times per week with organic lettuce. Young adult snails - in which reproduction endpoints are more sensitive to chemicals, including some endocrine-disrupting chemicals, than in fully grown snails [35] - were used. Snails of homogenous size (22.5 ± 2.5 mm) and age (4 ± 0.5 months) were sampled from the culture and acclimatised to test conditions (*i.e.*, similar to culture conditions, but with a higher food quantity provided per individual) during the 48 h prior to chemical exposure.

2.2.2. Tested chemicals and concentrations

Tributyltin hydride (Chemical Abstract Service [CAS] number 688-73-3) and triphenyltin chloride (CAS number 639-58-7) were dissolved in analytical grade acetone (99.9% purity) to prepare stock solutions (10 µg/µL). Final solvent concentration (100 µL/L) was homogenous among treatments, as recommended by the OECD [36](except for the water controls). Exposure media consisted of culture water contaminated with stock solutions. Organotin concentrations were chosen based on literature data in gastropods [17-18, 22, 37] and *L. stagnalis* [19, 21]. Range-finding tests in *L. stagnalis* were also performed under similar tests conditions [38-39]. Based on this information, the chosen nominal concentrations were 45 ng Sn/L, 100 ng Sn/L, 220 ng Sn/L, 480 ng Sn/L, and 1065 ng Sn/L for TBT, and 100 ng Sn/L, 215 ng Sn/L, 755 ng Sn/L, 1000 ng Sn/L, and 2626 ng Sn/L for TPT. To facilitate the quantitative comparison of TBT versus TPT effects, all concentrations were expressed in tin equivalent; concentrations could thus be compared on a molar basis.

2.2.3. Test design and biological endpoints

Six replicates (each with 5 snails in 1-L glass beakers) per tested concentration, water control, and solvent control were randomly distributed in the exposure room. Snails were exposed to toxicant for

only 21 d because this duration was sufficient to provide evidence of effects and assess various effective concentration (EC_x) values for the compounds tested in the proposed experimental conditions (as determined in pre-experiments). Test water was renewed with freshly contaminated medium to maintain exposure concentrations and adequate physicochemical properties of test water. Renewal rates resulted from a compromise between maintaining exposure concentration and avoiding too much stress to the snails. Independent studies showed that in our test conditions, TBT water concentration dropped rapidly (92% losses in 72 h), so that the TBT test medium was renewed every other day. The TPT was more stable (62% losses in 72 h), and thus the TPT test medium was renewed every 3 d. Tests were conducted at 20.5 ± 0.6°C and in a 14:10-h light:dark photoperiod as previously described [35]. Snails were fed daily *ad libitum* with organic lettuce rinsed with culture water, which is an adequate food source to support adult snail growth and reproduction [40].

Dead snails were counted and removed daily. Individual shell length was measured using a digital caliper at days 0 and 21 to assess a possible impact on growth. Effects on reproduction were estimated by monitoring the cumulated number of egg clutches per snail and the cumulated number of eggs per snail. Every day, clutches were counted, removed using a sharp-edged spoon, and photographed. The number and quality of the eggs were determined by observation of the photographs. Egg quality was assessed by determining the frequency of 4 types of abnormalities (Figure 2.1A–E): polyembryonic egg (the presence of several embryos per egg; unfertilised egg (the absence of embryo in the egg, which only consists of the eggshell and albumen; atrophied albumen (damaged eggshell containing an abnormally low albumen quantity); and single embryo (presence of a nondeveloping embryo, without an eggshell and without albumen). Polyembryonic and unfertilised eggs have been described before [41-43], and polyembryony has been shown to be a sensitive endpoint [41]. The 2 other abnormalities are described for the first time in *L. stagnalis*.

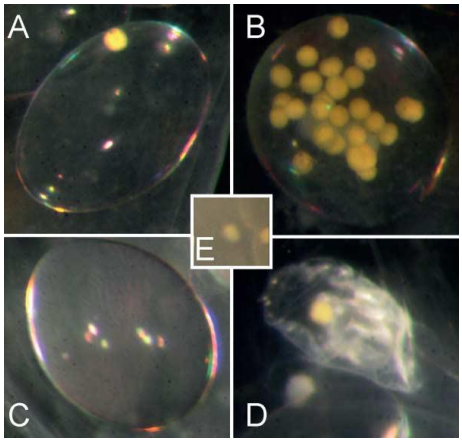

Figure 2.1. Different egg abnormalities observed in *Lymnaea stagnalis*. (**A**) normal egg; (**B**) polyembryonic egg; (**C**) unfertilised egg; (**D**) egg with atrophied albumen; (**E**) single embryonic cell.

2.2.4. Chemical analysis

Water was sampled in controls and every tested concentration at the beginning, middle, and end of the tests. Water was collected both 15 min after stirring stock solutions in clean water (*i.e.*, new exposure water) and just before water renewal (*i.e.*, old exposure water) to allow calculation of time-weighted average exposure concentrations. For each concentration and sampling date, three samples of 1 L were collected, which consisted of a mixture of 165 mL of water extracted from each exposure replicate. In the TBT experiment, mucus that had accumulated on the walls of the test beakers was also collected for chemical analysis. Samples were frozen until analysis of their MBT, DBT, and TBT and TPT content. Analyses were performed by coupled capillary gas chromatography/mass spectrometry, with a limit of quantification of 10 ng Sn/L equivalent [44].

2.2.5. Data analysis

Actual exposure concentrations were calculated as the time-weighted average of measured values over the test, using the formula proposed in [45]. Biological data were analysed with standard statistical analysis procedures, as described by the OECD [46], using Sigma-Stat (Jandel Scientific) and GraphPad Prism 5.0 software. Analyses of survival and reproduction data were based on observations for the six replicates, and analysis of growth data was based on individual length measurements. Analysis of abnormality frequency was based on the total number of clutches collected during the experiment. Statistical differences between water and solvent controls were determined using *t* tests or Wilcoxon tests. When a significant difference with water control was shown for one of the compounds, solvent controls were used as the reference in subsequent statistical studies for both compounds. Indeed, using the same type of controls for the calculation of EC_x values allows one to avoid confounding effects of the solvent. In other cases, water and solvent controls were combined.

Differences among treatments in survival, shell length, and cumulated number of clutches and eggs per individual were tested using Kruskal–Wallis tests, with Dunn's post hoc tests for survival and size and Dunnett's post hoc test for reproductive endpoints. Differences in the frequency of abnormal eggs were assessed using Mann–Whitney tests. All tests were performed with a $\alpha = 0.05$. In case of significant effects of TBT or TPT, different lethal concentrations (LC_x) or EC_x were calculated using a logistic regression model [47]. The 95% confidence intervals (CI) were simulated based on weighted residues to account for differences in variance across treatments and using 5000 bootstrap simulations. The Microsoft Excel macro REGTOX_EV7.0.6 xls was used for this purpose [48].

2.3. Results

2.3.1. Actual exposure conditions

The chemical preparation and contamination method resulted in exposure concentrations corresponding to 62.7 ± 14.2 % and 87.5 ± 17.6 % of the nominal TBT and TPT concentrations, respectively, at day 0. Water concentrations dropped rapidly and with different kinetics for TBT and TPT. Before water renewal, exposure concentrations had dropped to 35.7 ± 4 % and 18.1 ± 3 % of the nominal TBT and TPT concentrations, respectively. This resulted in time-weighted average concentration ranges of 19 ng Sn/L, 43 ng Sn/L, 94 ng Sn/L, 197 ng Sn/L and 473 ng Sn/L for TBT and 45 ng Sn/L, 74 ng Sn/L, 187 ng Sn/L, 265 ng Sn/L and 590 ng Sn/L for TPT. High TBT concentrations were also found in the mucus sampled after 21 days (*e.g.*, 1563 ± 638 ng Sn/L at the highest tested concentration). Both NBT and DBT were found in water, due to the degradation of TBT. Concentrations of MBT and DBT were not included in the calculation of TBT time-weighted average concentrations because their concentration were generally lower than the quantification limit, so they could be neglected.

2.3.2. Survival

Survival was 100% in water controls from both tests and 93% and 88% in the solvent controls from TBT and TPT tests, respectively. No significant mortality was recorded following exposure to TBT over the test duration. Exposure to the highest tested concentration of TPT (590 ng Sn/L) led to 100% mortality after 3 d of exposure ($p < 0.05$, Dunn's *post hoc* test), while lower concentrations did not significantly affect survival. The corresponding median lethal concentration (LC50) at 21 d was estimated at 436.1 ng Sn/L (CI: 308.1 – 433.6 ng Sn/L).

2.3.3. Shell size and integrity

Shell length at 21 d was significantly reduced in snails exposed to TBT concentrations exceeding 94 ng Sn/L (Figure 2.2A). A nonsignificant decrease was also observed in snails exposed to 43 ng

Sn/L. A careful inspection of snails showed that the apex of the shells was broken in some or all of the exposed individuals, leading a reduced shell length. Snails exposed to 0 ng Sn/L and 19 ng Sn/L generally did not suffer such injury. This finding suggests that broken shells were not experimental artifact but a consequence of exposure to TBT. Indeed, the frequency of harmed shells increased linearly with TBT concentration ($r^2 = 0.94$) and reached 100% at the highest tested concentration of 473 ng Sn/L (Figure 2.3A). Therefore, the reduction in shell length provides evidence for the effects of TBT on shell solidity. Analysis of size data only in snails that did not suffer shell injury highlighted a significant reduction in growth for TBT concentrations exceeding 94 ng Sn/L. As the growth effect was not very intense in the concentration range tested, no reliable EC_x value could be calculated. Damaged shells were observed at all TPT concentrations tested (but not in controls), suggesting that TPT also had an impact on shell solidity (Figure 2.3B). Damage frequency was between 50 and 70%, regardless of exposure concentration. No significant effect on growth was found with TPT (Figure 2.2B).

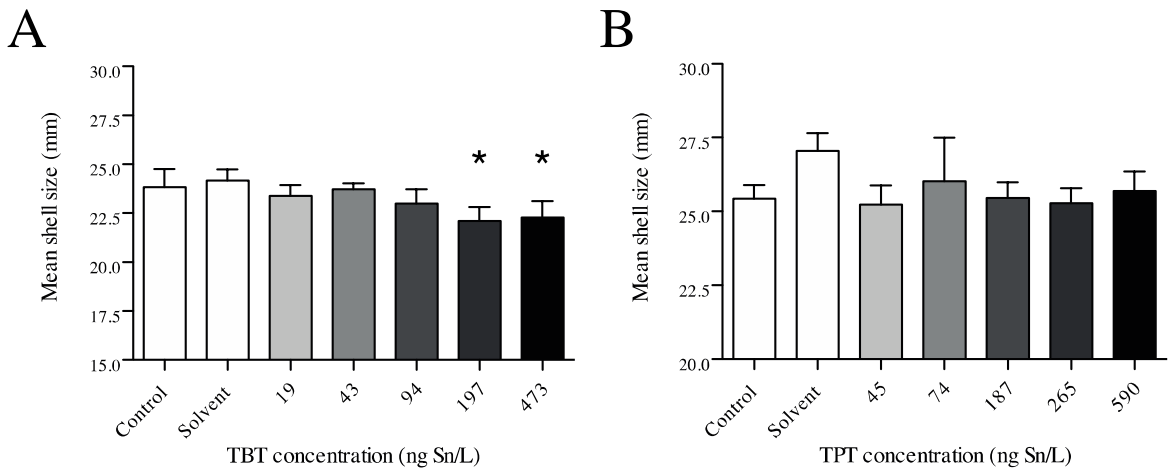

Figure 2.2. Mean shell size after a 21-d exposure to A) tributyltin (TBT) or B) triphenyltin (TPT). Error bars represent standard deviation over six replicates (★: $p < 0.05$).

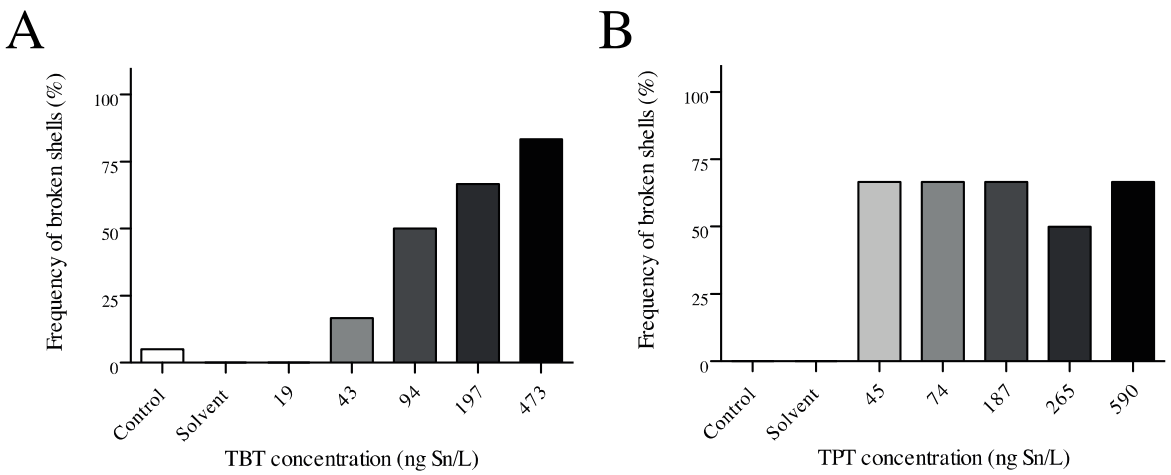

Figure 2.3. Frequency of broken shells observed over six replicates after a 21-d exposure to A) tributyltin (TBT) or B) triphenyltin (TPT).

2.3.4. Egg-laying behaviour

The time-course of effect was different in snails exposed TBT concentrations of 94 ng Sn/L or less: egg-laying occurred regularly but at a slower rate than in controls. For snails exposed to 197 ng Sn/L and 473 ng Sn/L, egg-laying ceased after 1 wk of exposure. The cumulated number of clutches produced per individual over 21 d decreased in all snails exposed to TBT compared with the water controls, from -24% at 19 ng Sn/L to -96% at 473 ng Sn/L (Figure 2.4A). A significant difference from solvent controls in the number of produced clutches ($p < 0.05$, Dunnett's post hoc test) was detected at 94 ng Sn/L. Exposure to the two highest TBT concentrations resulted in a severe reduction in egg-laying activity ($p < 0.001$, Dunnett's post hoc test). The corresponding median effective concentration (EC50) at 21 d was estimated to be 118.3 ng Sn/L (CI: 99.7 – 171.9 ng Sn/L).

In the TPT test, the time-course of effect was similar in snails exposed to all concentrations tested; snails regularly laid eggs, but the egg-laying rate was lower than in controls. Egg-laying activity was significantly lower in solvent control ($p < 0.05$, Wilcoxon rank sum test) than in water controls (Figure 2.4B). Solvent control was thus used as a reference for statistical tests for both TPT and TBT. Exposures to 265 ng Sn/L and 590 ng Sn/L caused a significant decrease ($p < 0.01$ and $p < 0.001$, respectively, Dunnett's post hoc test) in the cumulated number of clutches produced (-38% and -81% of the solvent control value, respectively). Based on these results, the estimated EC50 21-d value was 264.1 ng Sn/L (CI: 258.5 – 280.5 ng Sn/L).

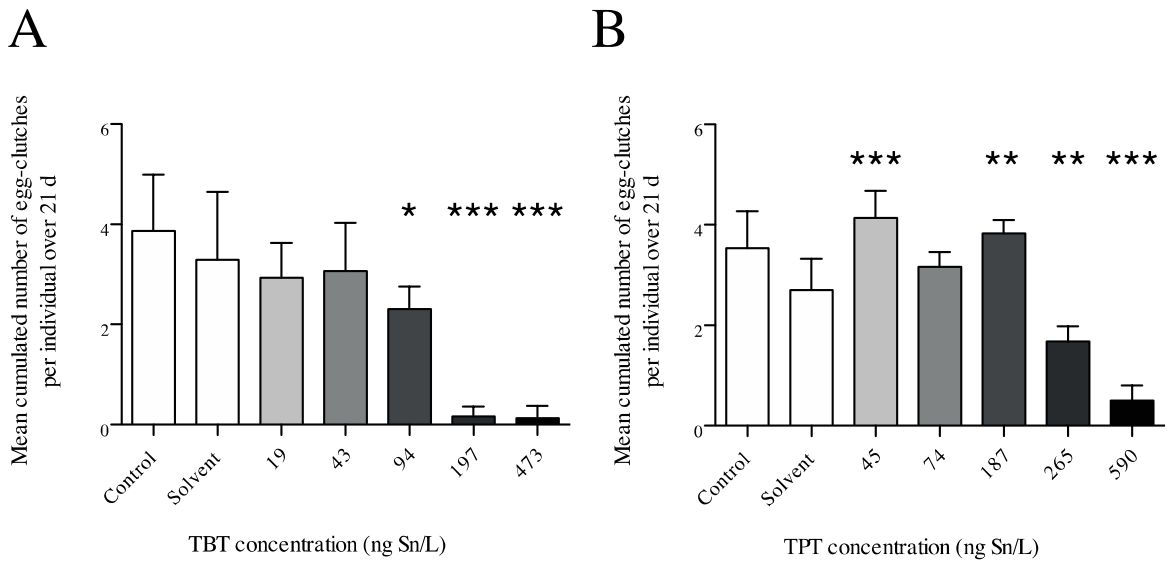

Figure 2.4. Mean cumulated number of egg-clutches laid per individual after a 21-d exposure to A) tributyltin (TBT) or B) triphenyltin. Error bars represent standard deviation over six replicates (★: $p < 0.05$; ★★: $p < 0.01$; ★★★: $p < 0.001$).

2.3.5. Fecundity

The effect patterns and estimated no-observed-effect concentration (NOEC) and lowest-observed-effect concentration (LOEC) values of TBT and TPT for fecundity were similar to the patterns observed for egg-laying behavior: the LOEC values were 94 ng Sn/L and 264 ng Sn/L ($p < 0.01$ and $p < 0.001$, respectively, Dunnett's post hoc test) for TBT and TPT, respectively.

Compared with water controls, a nonsignificant reduction in fecundity was already observed at the 2 lowest TBT concentrations and was particularly severe at the 2 highest tested concentrations (from -32% at 19 ng Sn/L to -97% at 473 ng Sn/L, as shown in Figure 2.5A). The corresponding EC50 21-d value was 106.2 ng Sn/L (CI: 84.4 – 125.6 ng Sn/L), which was not significantly different from the EC50 at 21 d found using oviposition as an endpoint.

A significant solvent effect on fecundity was again observed in the TPT test ($p < 0.01$, Wilcoxon rank sum test). Fecundity of the snails exposed to the two highest TPT concentrations (265 ng Sn/L and 590 ng Sn/L) was significantly reduced (-36% and -85% of the solvent control value, respectively) as shown in Figure 2.5B. The corresponding $EC_{50\text{-}21d}$ value was 263.9 [253.8 – 280.5] ng Sn/L, which was similar to the EC50 21-d found for oviposition.

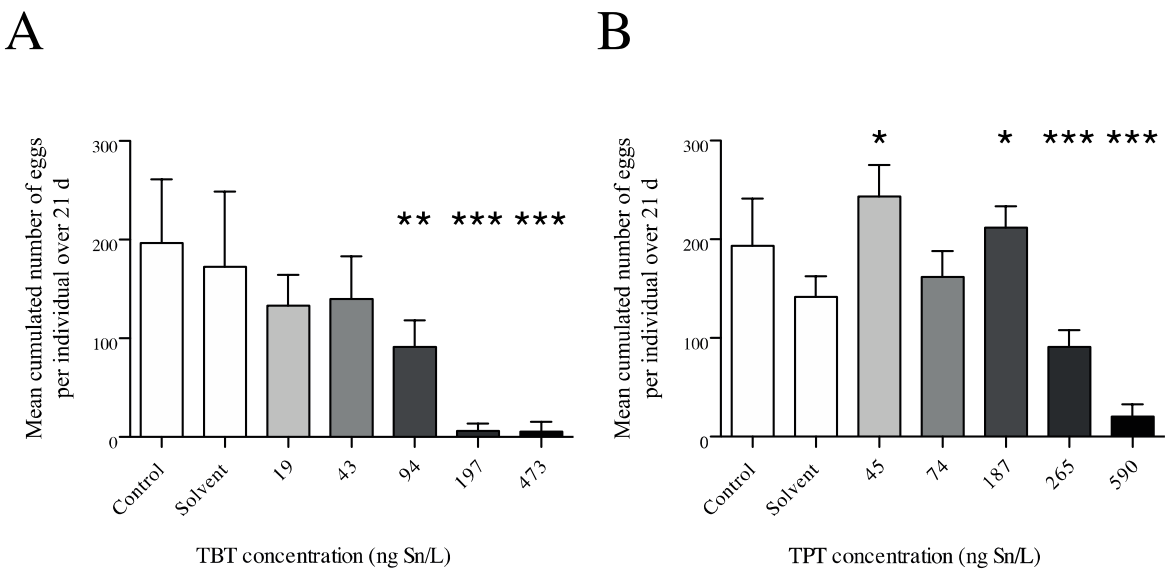

Figure 2.5. Mean cumulated number of eggs laid per individual after a 21-d exposure to A) tributyltin (TBT) or B) triphenyltin. Error bars represent standard deviation over six replicates (★: $p < 0.05$; ★★: $p < 0.01$; ★★★: $p < 0.001$).

2.3.6. Egg-abnormalities

Frequency of abnormal eggs per clutch increased over the test duration. After 21 d, it was significantly higher in snails exposed to TBT than in controls even at the lowest tested concentration of 19 ng Sn/L ($p < 0,05$ Dunnett's post hoc test). The effects of TBT on the frequency of unfertilised eggs, eggs with atrophied albumen and single-embryo were not significant. Frequency of polyembryony was the most interesting endpoint: it represented 65 % to 100% of observed abnormalities in the lowest and highest tested concentrations, respectively. It increased from +80% at 19 ng Sn/L to +177% at 473 ng Sn/L compared with controls (Figure 2.6). This increase was significant for all concentrations tested ($p < 0.05$, Dunnett's post hoc test). The corresponding EC50 at 21 d was 23.7 ng Sn/L (CI: 2.2 – 189.4 ng Sn/L). All eggs produced by parents exposed to 473 ng Sn/L exhibited polyembryony. In contrast, TPT induced no significant effects on the viability of eggs produced by exposed adults at the tested concentration range.

2.4. Discussion

2.4.1. Chronic effects of organotins in L. stagnalis

The TBT metabolite concentrations were very low (*i.e.*, below the quantification limit), which suggests that degradation might not be the most significant process contributing to the decrease in TBT concentration in water. Losses were probably mostly due to adsorption in mucus, for example. Indeed, high TBT concentrations — 3 to 5-fold higher concentrations than the measured peak concentration in water — were found in mucus sampled after 21 d. The TBT content in mucus most likely resulted from direct adsorption from water after the mucus was released and possibly, to a lesser extent, from elimination of TBT by snails via mucus. This adsorption might partly explain why only 62.5% of the targeted nominal concentrations were found in the water samples. This highlights the need to wipe mucus off test beakers between water renewals to limit adsorption of TBT on the test chamber walls, which reduces its availability to the snails.

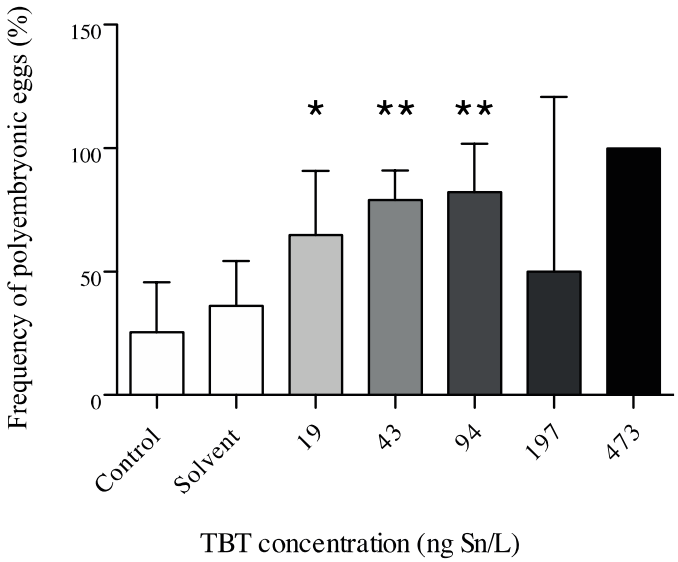

Figure 2.6. Frequency of the polyembryonic eggs (among the total number of abnormal eggs) found during a 21-d exposure to tributyltin (TBT). Error bars represent standard deviation over all abnormal egg produced (★: $p < 0.05$; ★★: $p < 0.01$).

Exposure of *L. stagnalis* for 21 d to TPT in the concentration range of 45 ng Sn/L to 590 ng Sn/L induced mortality at the highest tested concentration (LC50 at 21 d, 436.1 ng Sn/L), and a variety of sublethal effects at lower concentrations, that is, decrease in shell solidity at all tested concentrations, decrease in egg-laying activity and fecundity, endpoints that led to an identical EC50 at 21-d value of 264 ng Sn/L. Egg quality at the concentration range tested was not affected by TPT.

A 21-d exposure of *L. stagnalis* to TBT in the range of 19 ng Sn/L to 473 ng Sn/L did not result in significant mortality in the present study. Alternatively, Segner et al. [24] showed that adult survival was significantly reduced by TBT exposure, with an LC50 at 21 d of 290 ng Sn/L (nominal concentration), which is in contrast to both the present results and longer term studies from other authors. Indeed, no significant mortality was observed after 49 d and 84 d of exposure to 100 ng Sn/L (nominal concentration [21]), as well as after 56 d of exposure to the concentration range of 7 ng Sn/L to 181 ng Sn/L (unpublished data from a round-robin test). At this concentration range, significant mortality was only observed in a 170-d study in which snails were exposed to 410 ng Sn/L [19].

Exposure to TBT induced a decrease in growth at concentrations exceeding 94 ng Sn/L. It also induced a decrease in shell solidity in snails exposed to concentrations exceeding 43 ng Sn/L, which was also observed by Segner et al. [24] in 21-d tests for nominal concentrations exceeding 94 ng Sn/L. Exposure to TBT also induced a significant decrease in the egg-laying activity and fecundity of snails exposed to concentrations exceeding 45 ng Sn/L. This is consistent with previous results from Czech et al. [21], who observed a significant decrease in egg-laying at 100 ng Sn/L (nominal concentration) in a 49-d experiment. Overall, these data confirm the possible occurrence of reproductive effects in *L. stagnalis* exposed to TBT at environmentally relevant concentrations. Results obtained in our partial-life-cycle tests are in accordance with results obtained in other partial-life-cycle tests [21, 24] but differ from results obtained in the 170-d full-life-cycle test published by Leung et al. [19], in which animals were exposed from embryonic stage to adulthood. In the study of Leung et al. [19], adult fecundity was significantly modified at 41 ng Sn/L (NOEC), which was also the case in the present study (NOEC, 43 ng Sn/L). However, the effect pattern was quite different (S-shape dose–response curve in the present study vs inverted U-shape in the study

of Leung et al. [19], with an increase in fecundity at 41 ng Sn/L compared with controls). The magnitude of effects at 410 ng Sn/L was also different. Indeed, the fecundity was reduced by a factor of 10 compared with control in the study of Leung et al. [19], whereas it was reduced by a factor 100 in the present study. These results suggest that the test design might greatly influence the biological responses of *L. stagnalis*. Further studies are needed to assess the influence of test duration on the endpoint values in partial life-cycle tests and to compare the sensitivity of partial versus full life-cycle tests with *L. stagnalis*.

Egg quality was altered by TBT, which was best evidenced through the frequency of polyembryonic eggs in clutches from exposed parents. The increase in polyembryony frequency was already significant at concentrations that were lower than those affecting egg production by adults. This finding indicates that polyembryony is more sensitive to TBT than the other reproductive endpoints tested (*i.e.*, oviposition and fecundity, which are the common endpoints in reprotoxicity tests with snails), thus confirming previous findings with acetone [41]. Test endpoints/durations/protocols vary greatly among published studies on the effects of TBT in *L. stagnalis*, leading to a large variability in published NOEC values for some endpoints. This variability is also due to the lack of data on actual exposure concentrations in most published papers, which hampers a sound comparison of the published results. In this respect, the forthcoming standardisation of reproductive toxicity test protocols with *L. stagnalis* will help in deriving more reliable conclusions on the toxicity of TBT, TPT, and other types of chemicals.

2.4.2. Comparison of the responses of L. stagnalis to TBT vs. TPT

Differences in the sensitivity of *L. stagnalis* to similar concentration ranges of TBT and TPT were observed for most of the endpoints studied. The TBT concentrations tested (up to 473 ng Sn/L) had no significant effect on survival, whereas 100% mortality was observed at the highest tested TPT concentration (590 ng Sn/L), which suggests a higher toxicity of TPT than TBT to *L. stagnalis*. Results from a pretest with TBT confirmed that TBT has no effect on survival at 590 ng Sn/L. (The NOEC in this pre-test was 627 ng Sn/L, while the LOEC was 6270 ng Sn/L, with 100% mortality occurring at the end of the test; data not shown). A significant effect on snail growth was found with TBT, while no effect occurred due to TPT exposure. Because both TBT and TPT led to shell injuries, shell size might not be the most reliable growth indicator when assessing the effects of organotins on *L. stagnalis*. Indeed, decreased shell size due to an injury can be wrongly interpreted as an effect on growth. To avoid such misinterpretation, we recommend not using length data from individuals with broken shells for the statistical analysis of growth effects. Discarding such individuals from the analysis might lead to loss of a number of data and thus to a reduced statistical power when effects on growth are tested (*e.g.*, in the present study, 100% of snails were injured at the highest TBT concentration). Growth effects should rather be assessed through measurement of the soft body dry weight to avoid misinterpretation and maintain a sufficient statistical power. It is likely that the decrease in shell solidity was due to decalcification, which has already been observed for TBT in *L. stagnalis* [24] and other molluscs (*e.g.*, oysters [49]). Similarly, trialkylated tin compounds have been shown to interfere with calcification processes in mammals [49], which might also apply to *L. stagnalis* exposed to TPT.

Both compounds significantly reduced egg-laying and fecundity, but effective concentrations were lower for TBT than for TPT, as reflected by the EC50 21-d values for reproductive endpoints. The EC50s at 21 d based on fecundity data were equivalent to values obtained from oviposition data for TBT (EC50 21-d values of 106 ng Sn/L and 118 ng Sn/L, respectively; no significant difference as assessed through the overlapping of 95% confidence intervals) and for TPT (with a similar EC50 21-d value of 264 ng Sn/L and no overlapping of confidence intervals). These results suggest that both reproductive endpoints have a similar sensitivity to TPT and TBT in this particular experimental setup, although oviposition and fecundity are known to be under the control of two different hormonal pathways in *L. stagnalis* [32]. Exposure to TPT did not affect egg quality, whereas polyembryony often occurred in eggs produced by TBT-exposed snails. Polyembryony

thus exhibited different responses to TBT and TPT and was the most sensitive endpoint in the TBT test. The use of acetone as the carrier solvent did not significantly influence snail reproduction or egg quality in the TBT test but did significantly hamper all reproductive performances in the TPT test. Previous in-house studies showed that reproductive effects due to acetone occur from time to time in our experimental conditions. The reasons remain unclear; this may be a result of, for example, differences in solvent purity or snail sensitivity from one experiment to another. This highlights the need for additional studies devoted to the assessment of solvent effects in juveniles and adults of *L. stagnalis* to complement the recently published results on solvent effects in embryos [43].

Reasons for differences in sensitivity of *L. stagnalis* to TBT versus TPT remain to be elucidated. Differences in molecular structure of these organotins might be a relevant explanation. Indeed, it is assumed that the toxicity of organotins is more influenced by the alkyl substitutes than by the anionic substitutes [6]. A recent study in *Mytilus edulis* confirmed that alkylation of organotins influences their toxicity to molluscs [50]. Therefore, differences in the responses of *L. stagnalis* to TPT versus TBT are likely to be related to differences in their alkylation. However, it is not known how such differences in molecular structure might lead to different modes of action of these compounds in *L. stagnalis*. Previous studies highlighted the possible endocrine effects of TBT and TPT in gastropods [51-55]. Hormonal pathways involved in the response of various gastropods to TBT were investigated, focusing on the mechanisms of imposex induction in gonochoric marine species [51, 54, 56-58]. These studies highlighted the fact that imposex is related to an alteration in steroid homeostasis, mediated by inhibition of enzymes such as cytochrome-P450 aromatase or acyltransferase that are involved in biosynthetic steroid pathways [53, 58-59]. Other *in vivo* and *in vitro* studies showed that TBT and TPT are potent activators of nuclear receptors such as the retinoid X-receptors, leading to transcription of genes involved in steroid homeostasis [52, 60-63]. Other studies highlighted the effects of TBT and TPT on different components of the microsomal monooxygenase system of the bivalves *Mytilus galloprovincialis* and *Ruditapes decussata* and of the gastropod *Thais haemastoma* [64]. Finally, Lyssimachou et al. [17] showed that TPT was able to alter lipid metabolism in females of the ramshorn snail *Marisa cornuarietis*. However, it is not known to what extent these findings can be extrapolated to hermaphroditic species such as *L. stagnalis*. In the present study, reproductive effects of TPT occurred at quite high concentrations (*i.e.*, the LC50 at 21 d was only 2-fold higher than the reproductive EC50 at 21 d). Values for EC50 at 21 d were similar when estimated using fecundity or egg-laying data, indicating no relationship between the hormonal pathways involved in the control of these processes and the biological response. Furthermore, no significant increase in the frequency of abnormalities occurred in the offspring of exposed snails. Therefore, results of the apical reproduction test suggest that the reproductive impacts observed are probably linked to the toxicity of TPT to the snails rather than being a consequence of endocrine disruption. Alternatively, TBT concentrations that induced reproductive effects in *L. stagnalis* were much lower than the lethal concentrations reported in other studies conducted with this species [19, 24], and occurred at environmentally relevant concentrations. In addition, the frequency of polyembryony in the offspring increased in exposed snails. These results suggest that TBT might act as an endocrine disruptor in *L. stagnalis* as well. Even if apical endpoints might bring clues to possible modes of action of TBT and TPT in *L. stagnalis*, studies should be implemented to determine to what extent and for which compounds the observed deleterious effects of organotins are actually due to endocrine disruption [31-32].

2.4.3. Comparison of responses of the hermaphroditic snail vs. gonochoric species

Existing data, including the present results, have shown that TPT and TBT have different acute and chronic effects in various mollusc species. For instance, exposure of the parthenogenetic snail *Potamopyrgus antipodarum* to TBT and TPT in sediment biotests led to a significant reduction in reproductive output at environmentally relevant concentrations [8, 65]. Effect concentrations were lower and the intensities of effects on reproduction were higher in mud snails exposed to TPT

compared with those exposed to TBT. In contrast, acute toxicity occurred in snails exposed to TBT but not in those exposed to TPT. Based on these results, the effects of TPT and TBT in *P. antipodarum* are opposite to the effects we found in *L. stagnalis*. In another study, it was shown that females of the rock shell *T. clavigera* had a similar sensitivity to TBT and TPT (via direct injection in soft tissues), while males were more sensitive to TPT than to TBT [16]. Interestingly, it appears that three prosobranch snails with different habitats (freshwater, brackish water, and marine water) and different reproductive strategies (parthenogenesis, hermaphroditism, and gonochorism) were all responsive to organotin compounds, but their sensitivities were species- and sex-dependent, as reviewed by Ketata et al. [66]. Additional studies are required to provide explanations for differences in responsiveness and sensitivity to TBT and TPT in and between species.

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3. Considerations for test design to accommodate energy-budget models in ecotoxicology: a case study for acetone in the great pond snail *Lymnaea stagnalis*

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Abstract

Toxicokinetic-toxicodynamic (TKTD) modelling offers many advantages in the analysis of ecotoxicity test data. Calibration of TKTD models, however, places different demands on test design compared to classical concentration-response approaches. In this study we provide useful complementary information regarding test design for TKTD modelling. We present a case study for the great pond snail *Lymnaea stagnalis* exposed to the narcotic compound acetone, where the data on all endpoints were analysed together using a relatively simple TKTD model called DEBkiss. Further, we explored how standard toxicity tests data and additional data affect model calibration. DEBkiss described toxic effects on survival, growth, and reproduction over time well, within a single integrated analysis. Regarding the parameter estimates (*e.g.*, no-effect concentration, NEC), precision rather than accuracy was affected, depending on which data set was used for model calibration. Furthermore, we showed that the intrinsic sensitivity of snails to acetone stays the same across different life stages, including the embryonic stage. In fact, the data on egg development allowed to select a unique metabolic mode of action for the toxicant. We discuss the practical and theoretical considerations for test design to accommodate TKTD modelling, and hope that this information will aid other researchers to make the best possible use of their test animals.

3.1 Introduction

Toxicity tests, as any experimental test, are defined by their test design. Differences between test designs for a given species and toxicant may lead to large differences in the test results. Reviewing toxicity tests for the gastropod *Lymnaea stagnalis* exposed to tributyltin, Giusti and co-authors [1] showed that different protocols led to different values of the NOEC (no-observed effect concentration) and EC_x (estimated concentration causing x% effect), and even to conflicting conclusions about the adverse effects on reproduction for snails exposed to field-relevant concentrations. Reflecting the importance of test design, standard toxicity test protocols have been produced for a suite of test species; they define tailored test designs that ensure the reproducibility of toxicity tests within a species, which is essential when their results are to be used in ecological risk assessment.

A test design combines fixed "experimental conditions" and fixed "experimental effort." Experimental conditions comprise environmental factors that should be controlled during the test to ensure the well-being of the animals in the absence of toxic stress (*e.g.*, feeding conditions, light and temperature, water quality, density). These factors are thus mainly species dependent. Experimental effort comprises the number and range of test concentrations, the number of replicates, the number of animals per replicate, the test duration, the endpoints to be measured, and the frequency of these measurements over the test duration. The experimental effort is a complex component, as the optimal test design depends on the species, the toxicant, and especially on the foreseen analysis of the resulting data.

Ecotoxicity test data are routinely analysed using descriptive approaches to derive a summary statistic such as NOEC or EC_x. Current standard test protocols have been mainly designed to satisfy data analysis by hypothesis testing and concentration-response modelling. Yet, other modelling approaches are possible and recognized as valid in standard procedure for data analysis [2-3], such as mechanistic models that describe the toxicokinetics (TK) and toxicodynamics (TD) of chemicals (*i.e.*, TKTD models). TKTD models offer many advantages for the analysis of ecotoxicity tests [4-5]. Most importantly, they make optimal use of the information from the test data (*i.e.*, integrating observations over time, for different endpoints), and allow extrapolation to other environmental conditions and other exposure patterns. Furthermore, they hold promise for reducing the number of test animals, costs of toxicity tests, and for generating useful information to define risk mitigation measures. This makes TKTD models very appealing to risk assessors and stakeholders [6-7]. TKTD models place different demands on test design, but guidance on the most appropriate design to calibrate this kind of models is not available yet. This lack of guidance could be one of the major reasons why these models are not widely applied yet in ecotoxicology or environmental risk assessment.

The present study aims at providing useful complementary information for the analysis of toxicity tests data using a TKTD model. In this context, we exposed the great pond snail *L. stagnalis* to a narcotic toxicant (acetone). The experimental test was designed to optimise the calibration of a simple TKTD model from a small number of test animals, thus reducing experimental costs. We chose to work on the great pond snail because standard protocols with this species are currently under development [8], under the OECD test guideline development programme. A narcotic chemical was selected for the relatively simple mechanism of action, and to obtain results that are relevant for a large number of toxicants. Among existing TKTD models, only models that are based upon DEB (Dynamic Energy Budget) theory [9] have been included in ISO [2] and OECD [3] guidances for the analysis of ecotoxicity data (although they are hardly applied in risk assessment yet).

To promote the use of effect models, both for scientific as well as for risk assessment purposes, it is essential that models are not too complex, so that it ideally is possible to understand and use them without a background in modelling. Therefore, we used a simplified DEB model called DEBkiss [10] for the analysis of toxicity test data. Through a stepwise procedure, we evaluate the benefits of

using different types of data in the model calibration. We explore how standard toxicity tests data (*i.e.*, survival, size, and fecundity of adults) and additional data (*i.e.*, the same endpoints in sub-adults, and exposure-recovery patterns in both sub-adults and adults) affects model calibration. The optimal data set for calibration of the DEBkiss model is discussed in relation to the data already available from standard tests, to scrutinize the costs and benefits of additional experimental effort. Finally, we use the calibrated model to predict independent data for embryonic development (hatching success, hatching time, and snail size at hatching under toxic stress) to test the model's ability to describe this life-cycle stage as well.

3.2. Material and methods

3.2.1. Conceptual description of the DEBkiss model

A range of practical models has been derived from DEB theory, but most of the ecotoxicological applications rely on a simplification often referred to as DEBtox [11-13]. Because it contains fewer details about the animal's physiology than the standard DEB animal model [14], it is easier to parameterise and its calibration is less data demanding, which better suits the available data from toxicity tests. Here, we apply a more recent simplification, called DEBkiss [10]. In contrast to DEBtox, this model sports an explicit mass balance, which allows, for example, the implementation of starvation rules that respect mass and energy conservation. Furthermore, DEBkiss does not divide biomass into a reserve and structure component, and explicitly includes the embryonic stage. This model was earlier shown to provide a good description of the life cycle of *L. stagnalis* [10]. The structure, assumptions, and equations of the DEBkiss model used in this paper are presented in Appendix 1.

In TKTD frameworks, a toxicant first must enter the organism and reach a target site (TK), where it will then exert toxic effects (TD). Here we use a scaled first-order one-compartment TK model accounting for the effects of changes in body size and losses due to reproduction (Appendix 1). For embryos, TK is likely different than for sub-adults and adults, because of the different shape and nature of the interface between organism and exposure medium (the egg mass and egg membrane). Here, we simply assume instantaneous steady state between the egg and the environment, which is defensible in this case given the small size of the eggs, the fact that they were removed from the egg mass, and because of the rather rapid kinetics of acetone (a relatively high value of the elimination rate, see Table 3.1).

For toxicodynamics, we assume that the scaled internal concentration affects one or more of the DEBkiss model parameters that describe metabolic processes (*e.g.*, assimilation, somatic maintenance, maturation). An effect on a particular process directly translates into a specific change of the physiological responses of the snail over time (*e.g.*, growth and reproduction). The affected metabolic process(es), referred to as the metabolic mode of action (mMoA, see [15]), can thus be selected on the basis of the observed patterns in life-history traits. For a particular mMoA, a linear-with-threshold relationship is assumed between the scaled internal concentration and the affected metabolic process; the threshold is referred as the no-effect concentration (NEC). If several metabolic processes are altered by a toxicant, then each of them can have its own value for the NEC. However, we depart from the assumption that the observed changes in life history result from an effect on a single process. The internal concentration also increases the probability for the individual to die. This is modelled through a hazard rate, which is also linked to the scaled internal concentration using a linear-with-threshold relationship (fully equivalent to the case of simple stochastic death in GUTS [16]). The NEC for effects on mortality can be different than the one for metabolic effects (which affect sub-lethal endpoints).

3.2.2. Test species and experimental conditions

Snails (*Lymnaea stagnalis*, RENILYS strain) were cultured at the INRA Experimental Unit of Aquatic Ecology and Ecotoxicology (Rennes, France), under conditions previously described [17]. Culture medium was dechlorinated, charcoal-filtered tap water with the following physico-chemical characteristics: pH 7.4 ± 0.2, conductivity 428 ± 4 µS/cm, and dissolved oxygen 6 ± 1.2 mg/L. Snails were reared under a temperature of 20.9 ± 1.4°C and a photoperiod of 14:10 hours light:dark. Sub-adult (mean shell length of 18.6 ± 0.5 mm; no observed reproduction in the culture) and adult snails (29.3 ± 1.4 mm) were sampled from the laboratory culture and placed individually into glass beakers filled with 200 mL of the test medium (*i.e.*, culture medium spiked with acetone). Snails remained in the test medium all the time during the exposure, so that snail behaviour did not affect the hypothesis of constant exposure. The test medium was renewed twice a week in order to maintain its physico-chemical properties and to avoid fouling of test vessels (which were additionally cleaned with soft paper tissue at each test medium renewal). Animals were fed *ad libitum* with rinsed fresh organic lettuce. Prior to exposure, all snails passed a five-day acclimation period in test beakers, which ensured that they had recovered from sampling stress.

3.2.3. Test substance

Acetone CHROMASOLV (CAS number 67-64-1, purity ≥ 99.9%, Sigma-Aldrich) was used as test substance. Because this chemical is widely applied in toxicity tests as a carrier solvent for sparingly soluble substances, getting better insight on its unintended effects on organisms is desirable. Acetone was diluted in the culture medium by pouring the appropriate volume of acetone in each test beaker, using a micropipette, after removal of an equivalent volume of culture medium. The following nominal concentrations of acetone were used: 0.1, 0.3, 0.9, 3, 9, 14, and 18 mL/L. They were chosen based on the results of Bluzat et al. [18] and of pilot experiments we performed, aiming for a range from small to large effects on survival and reproduction. Acetone is a highly volatile chemical, and therefore, deviations from nominal concentrations were expected to occur between two renewals of the test medium. We expect volatilisation to be a first-order process, which means that the relative differences between the treatments are preserved. For simplicity, we will always refer to the nominal concentrations in the present paper.

3.2.4. Tests designs and endpoints

To obtain calibration data for the DEBkiss model, we conducted four partial life-cycle toxicity tests. The tests were performed simultaneously on groups of sub-adult and adult snails. Tests with sub-adults started with snails that were close to the size at maturity (approximately 20 mm). They became adults (onset of egg production) during the tests, but we keep referring to them as sub-adults in the present paper. Additionally, an embryo toxicity test was performed.

Exposure tests. Five sub-adult and five adult snails were exposed in isolation to acetone concentrations of 0.1 to 18 mL/L during 56 d. For the control group, 25 isolated snails were used instead of five, to obtain more precise estimates for the metabolic parameters of DEBkiss. Survival was monitored daily. Dead snails were removed from the beakers and stored at -20°C for dry weight measurement. Growth was assessed from shell length, measured once a week using a digital calliper, and from dry weight of soft bodies, determined only once at the end of the test (or when an individual died). Reproductive investment per snail was quantified through the cumulative dry weight of egg clutches. Test beakers were checked daily for the presence of egg clutches. They were carefully removed from the glass surface with a sharp-edge spoon and placed individually into plastic micro-tubes. The samples were stored in a freezer at -20°C prior to being freeze-dried for 24 h and weighed. To decrease the potential impact of isolation on the snails' reproductive performance, we allowed them to mate regularly during the tests. Snails were coupled (one "sub-adult" and one adult snail; always using the same couple) twice a week for 8 h, which duration sufficed to allow snails to mate [19]. Data sets obtained from sub-adult and adult exposure tests are hereafter referred as Je and Ae, respectively.

Recovery tests. Four adult and four sub-adult snails were exposed individually to acetone concentrations of 9 to 18 mL/L during 14 d, followed by a 42-d recovery period in clean medium. The control snails from the exposure test served as a control for the recovery test too. The same endpoints were followed as for the exposure test. Data sets obtained from sub-adult and adult recovery tests are hereafter referred as Jr and Ar, respectively.

Embryo test. To obtain data on embryonic development, we exposed snail eggs to a range of acetone concentrations from 0.02 to 18 mL/L according to the method developed by Bandow et al. [20]. Clutches, less than 24-h old and originating from adult non-exposed snails of homogenous age, were collected. Eggs from all collected clutches were extracted from their jelly-masses using a Pasteur pipette and placed into a Petri dish. Next, 12 eggs were randomly selected per treatment and placed into a 12-well plastic plate containing 10 mL of acetone exposure medium per well. The test duration was 21 d, the test medium was renewed twice a week, photoperiod was 14:10 hours light:dark, and the temperature was 22.4 ± 1.1°C. The following endpoints were monitored: time to hatch (checked daily after the first week of exposure), shell length at hatching, and the hatching success (embryos that did not hatch after 21 d were considered dead).

3.2.5. Stepwise model calibration strategy

Shell length is used as quantifier for body size of the snails over time, as it can be determined non-destructively. However, the state variable for body size in the DEBkiss model is dry body mass. Shell length is translated into dry body mass using a shape correction coefficient (δ_M) and the dry density of body tissue (d_V, see Appendix 1). The shape correction coefficient was determined as a mean value from 50 individuals (sub-adults and adults) of the control group. Dry-weight density of structure was obtained from an independent experiment performed in our laboratory, and was calculated as a mean value of 60 observations from non-exposed snails (Table 3.1).

The metabolic parameters of DEBkiss (Table 3.1) were estimated using the data from all control snails (sub-adults and adults) simultaneously. Indeed, experiments on adults alone do not provide sufficient information to identify the metabolic model parameters, owing to a lack of information on the growth rate and the start of reproduction. The only parameter that differed between the two age classes was the initial shell length. The initial size was taken as the mean of all shell sizes in each age class, and fixed in the analysis. The two yield coefficients (y_{BA} and y_{VA}) cannot be independently estimated from these data and were fixed to recommended values [10]. The complete set of estimated metabolic model parameters was subsequently fixed in the analysis of the data for the exposed snails.

Toxicological parameters were estimated by simultaneously fitting the data on survival, growth (shell length and dry soft body mass), and reproduction for all exposed snails (using nominal concentrations for exposure). To account for the variability of snail sizes between treatments, initial shell size was fixed to the mean size for each treatment, as described for control snails. The observations on dry soft body mass of snails indicated that animals shrank during exposures to higher concentrations of acetone. This implies that body tissue is used to pay maintenance costs (see Appendix 1), and therefore, we were able to estimate the yield of assimilates on structure, y_{AV}. To avoid unrealistic estimates, we restricted y_{AV} between 0.05 and 0.95.

The model was implemented in Matlab 2010b, and parameter values were estimated by maximising the overall likelihood function [11] (using square-root transformation of data for the continuous endpoints). The 95% confidence intervals on parameter estimates were generated by profiling the likelihood function [11].

To investigate what experimental design would be most suitable for model calibration, we calibrated the model using the data from each experiment separately, or in combination. This generated nine calibration and model parameter sets: Je, Jr, JeJr, Ae, Ar, AeAr, JeAe, JrAr, and JeJrAeAr. To test how the different parameters sets predict different data sets, we used each parameter set to predict the observations in each data set (comparing the log-likelihood value of the

prediction to the best possible value for that data set). The most likely mMoA of acetone was selected based upon the best model fit to all data simultaneously (JeJrAeAr), and fixed for the eight subsequent calibration sets. Fitting the model to different calibration sets suggested different possible mMoA. However, the most probable mMoA is the one that allows integrating effects on all monitored endpoints, as reflected by the JeJrAeAr calibration set. The correctness of the mMoA selected using this calibration set was confirmed by the observed effects on embryo development.

The data for effects of acetone on embryonic development were not used to fit the model, but only to test whether the parameter values from a fit on sub-adult and adult data suffices to predict toxicity to the developing egg. In light of previous model analyses for great pond snail eggs [10], we assumed an initial lag phase of 2.5 d in the embryonic development and tuned the specific assimilation rate of the embryo to match the observed hatching time in the control. The initial dry mass of a single egg (W_{B0}) was taken as 0.15 mg [10].

3.3. Results and discussion

3.3.1. Fitting control data

The DEBkiss model provided an excellent fit to all of the data for all control snails simultaneously (Figure 3.1). This supports the assumption that sub-adults and adults can be described by the same parameter set for basic physiology (Table 3.1). The tight confidence intervals show that the parameters values are well defined from the available data.

Table 3.1. Parameter values as estimated based on best fit to test data with *L. stagnalis*. Parameters δ_M and d_V were directly calculated from length data of both sub-adult and adult snails. The values of the toxicological parameters are calculated using nominal concentrations for exposure.

Metabolic model parameters	**Symbol**	**Value**	**Unit**
Specific maximum assimilation rate	J_{Am}[a]	0.187 (CI: 0.170-0.208)	mg/mm^2 d
Specific maintenance rate	J_M^v	0.0106 (CI: 0.00956-0.0119)	mg/mm^3 d
Allocation fraction to soma	κ	0.619 (CI: 0.606-0.627)	-
Shell length at maturation	L_{wp}	25.2 (CI: 25.0-25.6)	mm
Blank hazard rate	h_0	$1.11\cdot10^{-3}$ (CI: $0.184\cdot10^{-3}$-$3.39\cdot10^{-3}$)	1/d
Shape correction coefficient	δ_M	0.367 (SD: 0.0293)	-
Dry-weight density of structure	d_V	0.105 (SD: $5.90\cdot10^{-3}$)	mg/mm^3
Dry weight of a freshly-laid egg	W_{B0}	0.15[a]	mg
Yield of egg buffer on assimilates	y_{BA}	0.95[a]	mg/mg
Yield of structure on assimilates	y_{VA}	0.8[a]	mg/mg
Scaled functional response	f	1[a]	-
Toxicological model parameters			
Elimination rate constant	k_e	0.928 (CI: 0.754-1.16)	1/d
NEC for survival	c_{0s}	13.5 (CI: 12.6-13.8)	mL/L
Killing rate	b	0.0349 (CI: 0.0193-0.0574)	L/mL d
NEC for metabolic effects	c_0	0.879 (CI: 0.807-0.905)	mL/L
Tolerance concentration	c_T	26.2 (CI: 25.2-26.8)	mL/L
Yield of assimilates on structure	y_{AV}	0.397 (CI: 0.299-0.533)	mg/mg

[a] Parameter values are not calibrated but fixed to the suggested values [10].
CI = 95% confidence interval; SD = standard deviation; NEC = no-effect concentration.

The DEBkiss model was previously calibrated for the great pond snail by Jager et al. [10], using other experimental data from the same laboratory as for the present study. In contrast to the earlier

findings, in the present study, snails start to invest into reproduction at a higher body dry mass (79 vs. 67 mg, which corresponds to 25.2 and 21.4 mm shell length, respectively). This discrepancy might relate to differences in experimental set up between the studies. Indeed, snails were maintained in groups of five previously [10], whereas they were kept isolated in the present study. *L. stagnalis* is a simultaneous hermaphrodite which reproduces preferably by outcrossing. However, in absence of conspecifics, selfing occurs. Kept in isolation, snails may delay the egg laying [21], which might be a strategy to ensure lower inbreeding depression [22]. Snails from our study were maintained in isolation but were allowed to mate twice a week, which might constitute a sub-optimal mating frequency. Differences in soft body dry weight and size at maturation might also result from variation in life-cycle traits among snails (Appendix 1, Figures A2-4).

3.3.2. Fitting all treatments together

To select a mMoA, and to obtain a reference set of toxicity parameters, we fitted the model to data from all treatments together, fixing the metabolic parameters to the values obtained in control conditions (Table 3.1). The corresponding model fit for the exposure tests only is shown in Figure 3.1A-H; fits on the recovery tests are presented in Appendix 1, Figure A5. A range of mMoAs can be applied in the DEBkiss model to explain the effects of stressors on the life history of an organism [23]. The time course for growth and reproduction in the different treatments indicates which mMoA(s) most likely underlies the observed toxic effects. In the present study, the data were described almost equally well when assuming that acetone decreases assimilation (either by decreasing assimilation efficiency or feeding rate, resulting in a lower value of f), or when acetone increases the costs for both somatic and maturity maintenance by the same factor (higher value of J_M^v). This is a quite common situation as both affected metabolic processes produce similar patterns of effects post hatching [24]. Here, we selected effects on assimilation efficiency because this mMoA was consistent with the observed effects on embryonic development (as explained in the section “Predictions for embryonic development”).

The model described the toxic effects over time well on all endpoints, even though only a small number of animals were used for calibration. The fits to data on survival (Figure 3.1A, B) and dry weight of soft body (Figure 3.1E, F) are less satisfactory, although it must be considered that there is only one data point per snail over the whole test duration (for survival, the time of death is the relevant endpoint).

We observed a hormetic response in the exposure tests. In the tests with sub-adults, an increase in growth and reproduction compared to controls was observed, but not in the same treatments (0.1 and 0.9 mL/L, respectively). Adult reproduction was also stimulated at the lowest concentrations. This indicates that low concentrations of acetone enhance snails performance, although this could well be an experimental artefact (see [25].)

The data show that sub-adult snails shrank at 18 mL/L and adults shrank at 14-18 mL/L of acetone (Figure 3.1). Although the model predicted adult shrinking well, sub-adult shrinking is generally underestimated. The reason why the model does not capture adult and sub-adult shrinking well with the same parameter set is unclear. This indicates that the model assumptions about starvation in *L. stagnalis* (see Appendix 1) require further dedicated research.

In DEBkiss, toxic effects relate to the actual (scaled) internal concentration. Once snails are transferred to clean medium (in the recovery tests), the internal concentration will decrease over time, and thereby the effects. If an organism does not die, it is expected to fully recover after a certain period of time. The recovery patterns in the present study are well captured by the model, which shows that the assumption of full reversibility of effects on all endpoints holds for this compound (Appendix 1, Figure A5). To test assumptions about reversibility of effects, application of TKTD models is essential [26].

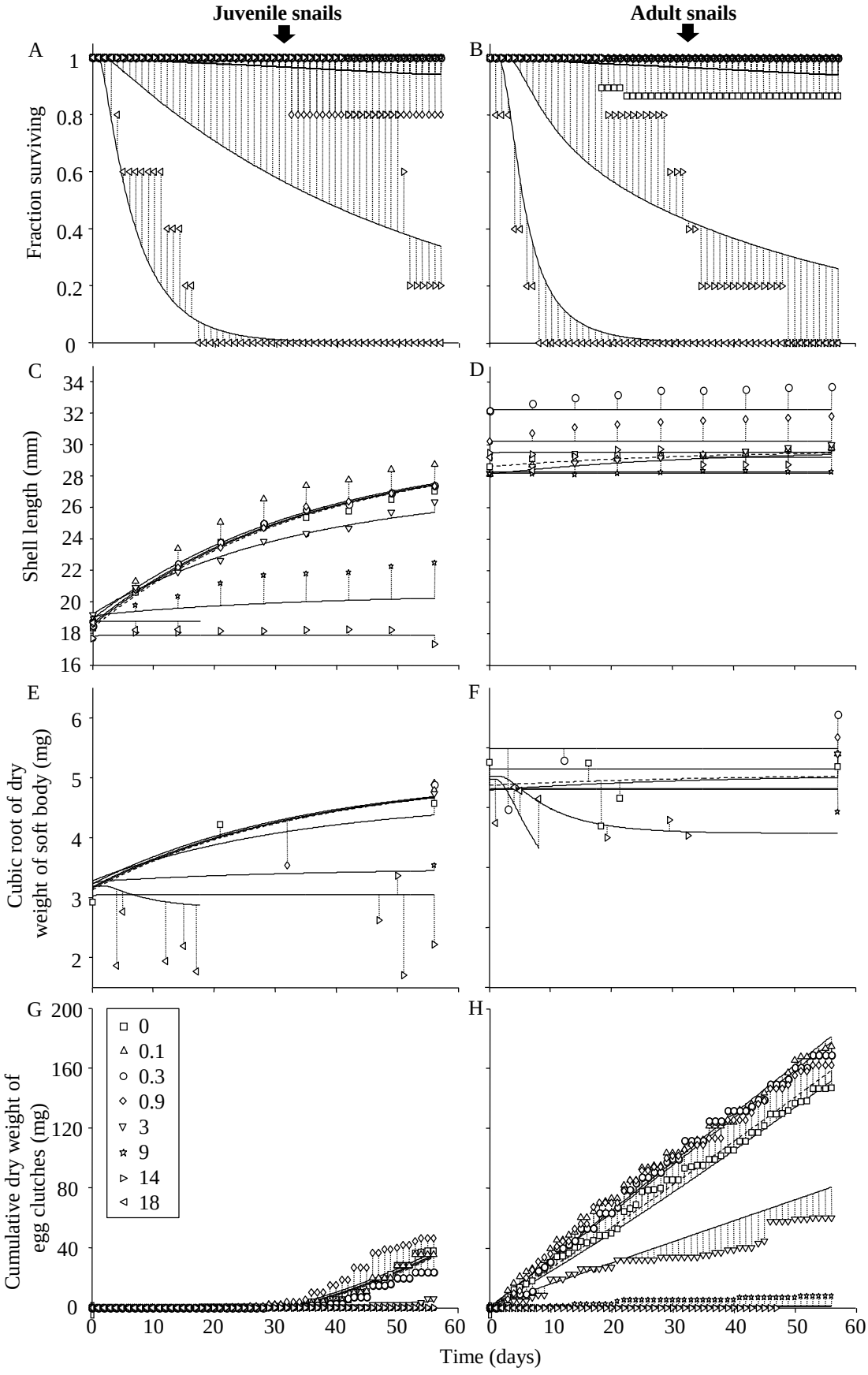

Figure 3.1. Model fits to effects of acetone in the exposure test on *L. stagnalis*. Fits to the following endpoints are shown: (A, B) survival, (C, D) shell length, (E, F) dry weight of soft body, (G, H) cumulative dry weight of egg clutches. Dotted and solid lines represent model curves for control and exposed snails, respectively. Symbols represent experimental data from different exposure concentrations of acetone (mL/L).

3.3.3. Comparing adequacy of various data sets for calibration

Next, we compared the values of toxicological parameters obtained by fitting different cross sections of the data set for exposed snails, to investigate the value of different data types for model calibration. Most of the parameter values are shown in Figure 3.2, while a complete set of parameter values is presented in Appendix 1, Figure A6. Interestingly, the parameter values are similar (as shown by overlapping confidence intervals) regardless of which data set was used for model calibration (Figure 3.2). The chosen calibration data set affects precision more than accuracy. Indeed, the confidence intervals of the toxicological parameters were narrowest when the model was calibrated on all data (the JeJrAeAr data set), which suggests that the data collected from all experiments provide a consistent picture of the toxicity of acetone. Overall, this analysis shows the robustness of the parameter estimation, as very different experimental sets lead to similar estimates for most parameters.

The best estimates for the NECs for survival and sub-lethal effects (Table 3.1) differ by a factor of 15.3, which ties in nicely with the 10-fold difference between commonly used trigger values of the Toxicity Exposure Ratio in aquatic risk assessment (*i.e.*, 10 for sub-lethal vs. 100 for lethal effects [27]). The best estimate for the NEC values differs only slightly (overlapping confidence intervals) between the different calibrations sets, which indicates that model can robustly identify NEC values from very different data sets.

For the yield of assimilates on structure, there is a discrepancy between estimations obtained from the different data sets: data sets with adults only result in high yields, whereas sub-adults favour low values (Figure 3.2). This yield coefficient is involved in the shrinking process, so this discrepancy is in line with the observation that sub-adult shrinking is poorly captured by the model.

Subsequently, we compared how well the model parameters obtained from one data set would predict the observations in another (Figure 3.3). The general pattern is that a model calibrated on one age class, regardless of the exposure type, predicts best the other data of that same age class. The model calibrated on the most complete data set within the age class (*i.e.*, JeJr, AeAr and JeJrAeAr) best predicted the less complete sets within the same class (*i.e.*, Je and Jr, Ae and Ar, JeAe, and JrAr). However, the precision with which data were predicted varies across the calibration sets. This analysis highlights the sensitivity of the model predictions towards differences in the values of toxicological parameters; small deviations in these parameters values due to a different calibration data set (Figure 3.2) may lead to significant differences in goodness of fit for predicted data sets (Figure 3.3). Given the difficulties in estimating y_{AV}, as discussed above, it is likely that the inadequacy of the starvation rules is the main culprit here.

3.3.4. Predictions for embryonic development

The metabolic parameters from Table 3.1 can be used to predict embryonic development in uncontaminated conditions. However, these parameters would lead to very short hatching times, as was also observed earlier [10]. Apparently, snail embryos do not grow much during the first few days, and then grow at a lower rate than expected from the growth curves of sub-adults and adults. The first observation can be captured by a 2.5-d lag phase in development [10], but for the slow rate of development, the only explanation within DEBkiss is that the specific rate of utilization of the egg buffer is lower than the specific assimilation rate of sub-adults and adults. Here, a factor of 0.4 explains the observed hatching time in the control (in [10], a factor of 0.5 was used). This slower assimilation rate for embryos might be related to the apparent food limitation in early sub-adults of the great pond snail [24]; sub-adults up to a certain shell size grow more slowly than later in life, which relates to food quality and/or feeding rates. The predicted absolute shell size at hatching (not shown) is much larger than the observed values. This likely results from using the same shape coefficient for hatchlings as for adults (shells of hatchlings are less pointy). For this reason, in Figure 3.4A we present shell size relative to that of control snails.

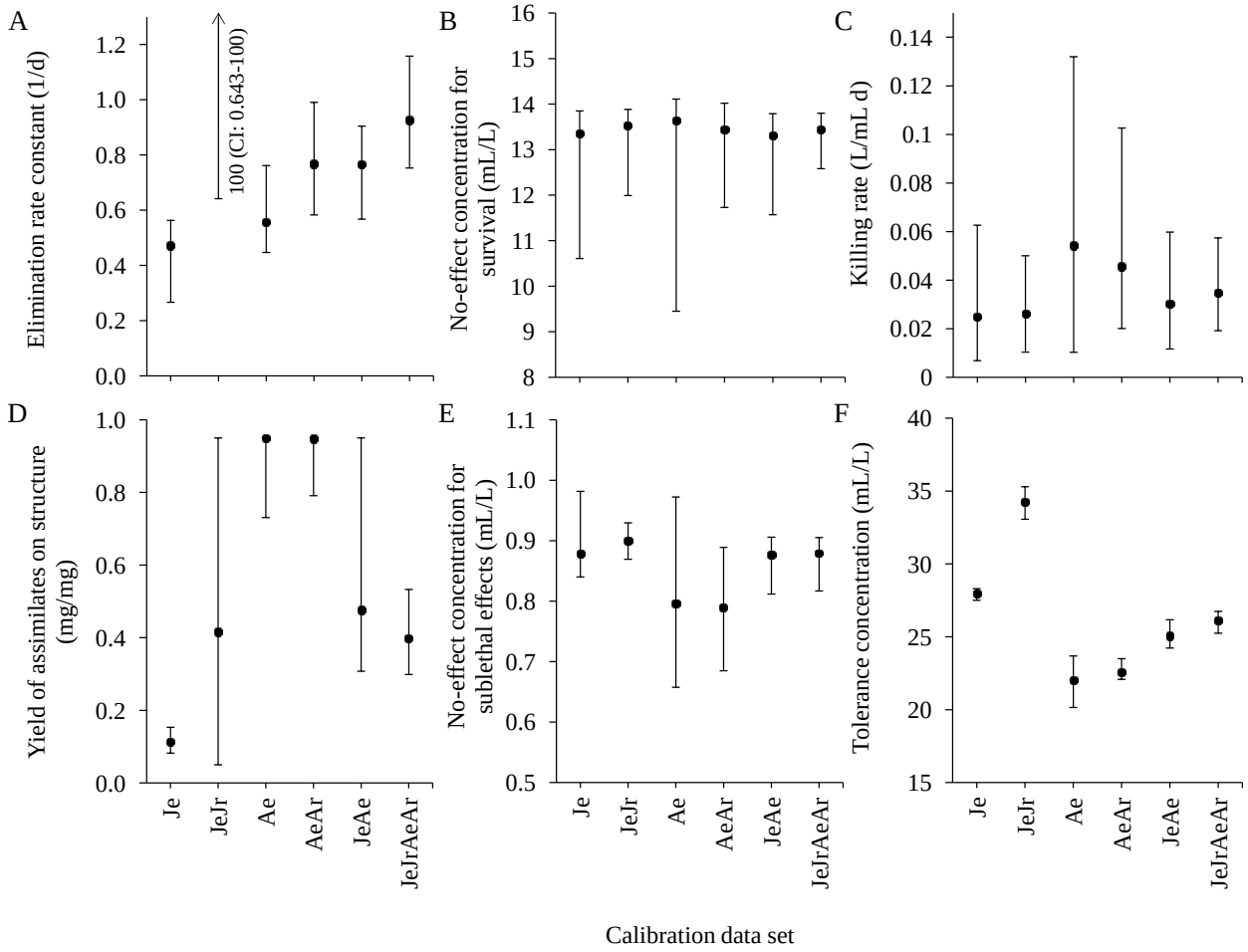

Figure 3.2. Toxicological model parameters values estimated from various calibration data sets. Error bars represent 95% confidence intervals. The arrow in Figure 3.2A indicates a very high parameter value (instantaneous steady state).

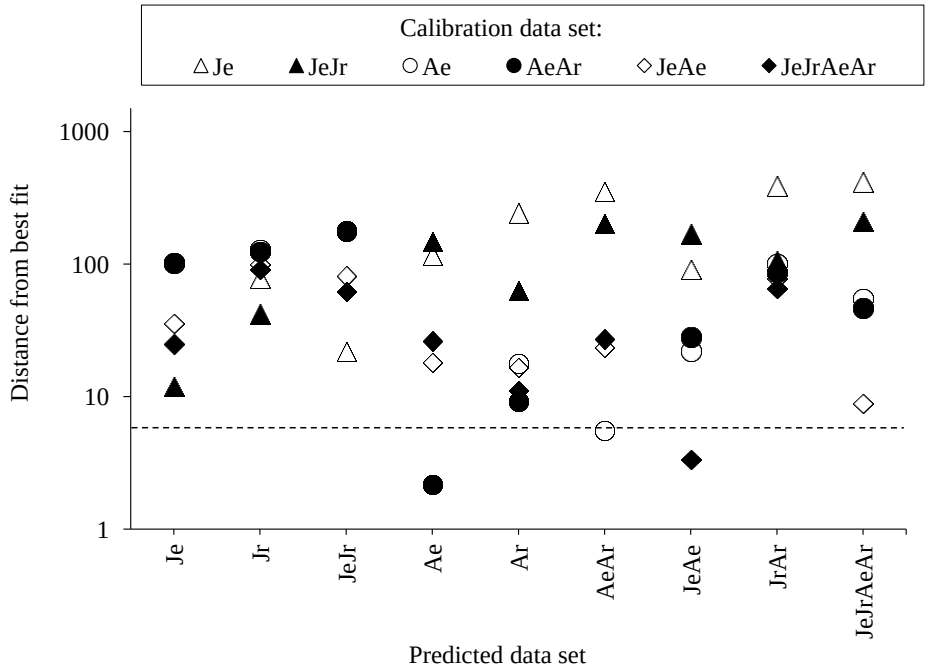

Figure 3.3. Data predicted from different calibration sets. The distance from the best fit represents the absolute difference in log-likelihood values between the prediction using a specific calibration set and the best possible fit (calibrating on the predicted data set itself). Lower values imply better predictions. Broken line represents the critical value of 6.3, above which predictions are significantly different from the best possible fit in a likelihood-ratio test at the 95% confidence level.

The predictions for the embryonic endpoints as a function of the acetone concentration are provided in Figure 3.4A-C for the three mMoAs that explain sub-adult and adult data well: a decrease in feeding rate (*i.e.*, a decrease in the utilisation rate of assimilates by the embryos from the egg buffer), a decrease in assimilation efficiency, and a combined increase in somatic and maturity maintenance costs. The first two mMoAs are both reflected in a change of the value of the parameter *f* for the embryo, just as done for sub-adults and adults. However, in contrast to sub-adults and adults, food is not supplied *ad libitum* for the embryo, which leads to differences in development between the two mMoAs. Decreasing the utilisation rate of the limited egg buffer means that the non-assimilated food is available for later use, whereas a decrease in the assimilation efficiency implies an increase in the overhead costs and thus that a larger part of the egg buffer is dissipated. Interestingly, the hatching time and hatchling size allowed distinguishing between various possible mMoAs of acetone. A decrease in the assimilation efficiency clearly provided the best explanation for the observed patterns. Selecting the most probable mMoA for a toxicant is important from a scientific point of view but also for practical purposes (especially in the extrapolation to other environmental conditions), but is a recurring problem in the application of DEB models in ecotoxicology [28-29]. In many cases, standard toxicity tests do not provide enough information to differentiate between various mMoAs (especially assimilation and maintenance). Focusing on goodness-of-fit can be dangerous as the best-fitting mMoA may well depend on rather arbitrary assumptions in the statistical approach (*e.g.*, transformation of the data). Here, we demonstrate how effects on egg development can aid the selection of the mMoA.

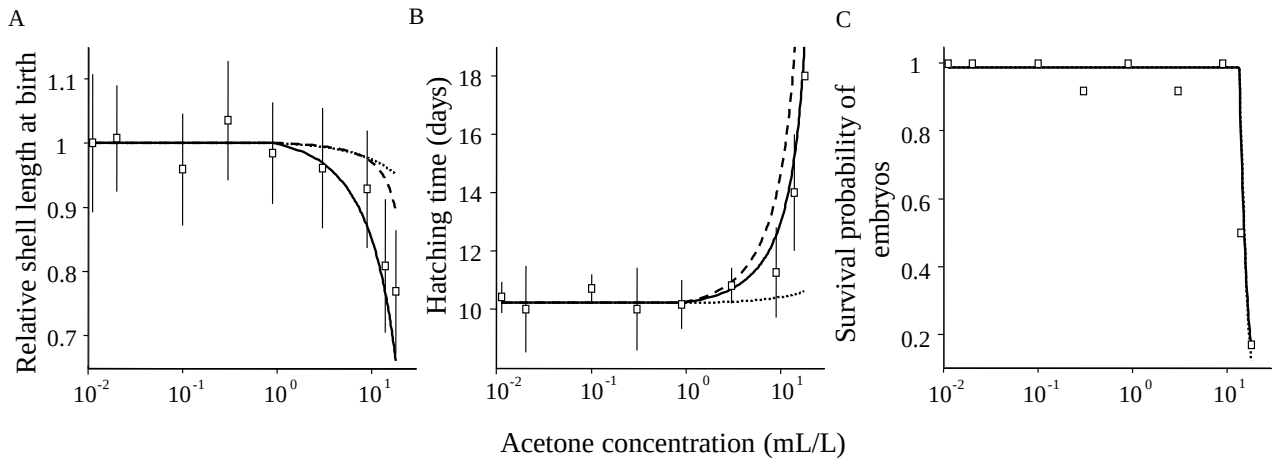

Figure 3.4. Predictions of embryo responses to acetone: A) shell length at birth relative to control, B) hatching time, C) survival probability at d 14 of the test. Squares represent data points. Error bars represent standard deviations of the data. Curves represent predictions with different metabolic mode of action (mMoA): decrease of assimilation efficiency: solid line; decrease of feeding rate (*i.e.*, utilisation of the egg buffer): broken line; increase of somatic and maturity maintenance costs: dotted line.

Prediction of the toxic effect of acetone on embryonic development from the parameter values in Table 3.1, suggests that embryos are not intrinsically more or less sensitive than sub-adults and adults to this compound (where intrinsic sensitivity is defined as the relationship between the internal concentration and a metabolic process [26]). In addition, the predictions show that the predicted survival probability for the embryo corresponds well with the observed hatching success. Even though we did not check whether non-hatched embryos were alive or not, the acetone treatments where the NEC for survival is exceeded result in a drop of the hatching success with the same factor as the predicted survival probability. Finally, we could predict both embryonic development and sub-adult and adult life-cycle traits by the same mMoA: a decrease in assimilation efficiency. This supports the DEBkiss interpretation of embryonic development (where embryos assimilate in the egg) in contrast to the standard DEB model assumption (where embryos do not assimilate but mobilize their reserve), at least for the great pond snail. It should be noted that toxicity tests on eggs extracted from their jelly-mass [20] are inappropriate to realistically assess the

effects on embryos of *L. stagnalis* in the field. Yet, to design a simple TKTD model, it is important to remove the complicating factors that the egg mass contributes to TK but also to the development of the embryos [30].

3.3.5. Consequences for experimental design

Overall, DEBkiss described the effects of acetone well, with rather similar parameter values irrespective of the calibration data provided. In the present study, we estimated the metabolic parameters using the control data for sub-adults and adults together. It must be stressed that data from sub-adults only, and especially adults only, is not sufficient to parameterize TKTD models such as DEBkiss because neither data set by itself contains enough information to first calibrate the model for control (uncontaminated) conditions. To estimate the metabolic parameters, data on the growth curve, the start of reproduction, and the reproduction rate are required, which implies testing with sub-adults and adults (or following sub-adults well into adulthood). Another limitation of using data on adult snails only is that it becomes more difficult to identify a single, most probable, mMoA for the toxicant: effects on the growth curve are essential to distinguish between the various options. Furthermore, as adult snails are close to their maximum size at the start of the experiment, a toxic effect on assimilation or maintenance will rapidly induce starvation, making it difficult to decide whether a poor fit is caused by an incorrect mMoA or unrealistic starvation rules.

Adding information on recovery made surprisingly little improvement on the precision of the parameter estimates. Only the confidence intervals of the NEC and the killing rate were slightly narrower when data on recovery was included. On the other hand, it has been shown that in some cases including exposure-recovery treatment can substantially improve the precision of some parameter estimates [31]. From a risk assessment perspective, it might be important to test the reversibility of effects, particularly for plant protection products, whose application often result in highly time-variable exposure. In that case, it is important to assess whether or not individuals fully recover between pulses or if damage accumulates [32].

We further show that the additional determination of dry weight at the end of the test is extremely helpful to establish whether snails shrink in the treatments, which is clearly not possible from shell size only. As the dry weight is a destructive measurement, it cannot be used to monitor changes in weight of exposed animals along the test. It would be interesting to investigate whether measuring wet body weight (including the shell), at least several times during the test, could provide a useful source of information. Obtaining weight data also helps to decrease the options for the mMoA in some cases (only effects on assimilation and somatic maintenance will induce shrinking).

Reproductive performance in toxicity tests is usually measured as number of eggs. However, DEBkiss specifies a reproductive mass flux as the primary output of the model. Thus, it is more accurate to calibrate the model directly on data for dry mass of clutches than to count the number of eggs and use an average dry mass per egg. Weighing egg clutches instead of counting eggs also requires less effort. However, counting the eggs in at least some clutches is important to identify possible effects on the investment of assimilates per egg (which would be essential to assess the population consequences of the stressor).

The standard OECD protocol under development for *L. stagnalis* includes 30 snails per concentration, which is recommended when a descriptive analysis of the data is foreseen, given the large variability of the snails. Calibrating the DEBkiss model makes no strict demands on the number of individuals; increasing the number of individuals increases the reliability of the analysis and leads to narrower confidence intervals on the parameters. The number of animals thus has to be chosen based on the desired precision and the variability in the test population. Here, we demonstrate that even with five animals per treatment we were able to estimate the NECs with good precision (Figure 3.2). In our experimental set up, we followed individual snails rather than keeping them in groups. Even though we did not make use of this property in this study, it allows for

analyses accounting for inter-individual variability (see [33]), which could further increase the efficiency of model calibration.

TKTD models also make no strict demands on the number of test concentrations, although a range of effects (from none to large) is recommendable to allow identification of the toxicological parameters and a unique mMoA. To calibrate such models, it is however absolutely essential to have observations over time for multiple endpoints (body size, reproduction, survival). Statistical power can most easily be increased by increasing the number of observations over time.

In summary, the most appropriate test design to calibrate the DEBkiss model would be to follow all endpoints over time from the same cohort, starting from the sub-adult stage and continuing well into adulthood. For body size, shell length and dry body weight (at the end of the test) are suitable endpoints, although additional wet weight measurements might be useful. For reproduction, the dry weight of clutches is most appropriate, with additional counting of the number of eggs in some clutches. To select a most appropriate mMoA, data on some additional endpoints might be helpful, such as the feeding rate [34] or data on embryonic development, as demonstrated in this study. Testing recovery does not specifically help the estimation of model parameters (at least in this case study), but is very helpful to demonstrate the reversibility of effects.

3.3. Conclusions

The DEBkiss model can be used to explain the toxic effects of acetone in *L. stagnalis* on survival, growth, and reproduction over time, in one integrated analysis. The model also provided a consistent explanation for effects on embryonic development. In fact, the embryo data enabled us to decide upon the most appropriate mMoA, which opens up possibilities to include embryonic effects (which are often ignored in ecotoxicology) as an integrated element in test design. We also showed that the intrinsic sensitivity of snails to acetone stays the same across all developmental stages, which may well be a feature of narcotic chemicals in general.

Clearly, calibrating TKTD models such as DEBkiss place different demands on test design compared to classical concentration-response approaches. The draft OECD protocol for the great pond snail is thus not useful to calibrate TKTD models, and also not intended for that purpose. Here, we discuss a range of practical considerations for test design relating to the calibration of TKTD models. However, one should realize that different toxicants may require different designs. For substances other than narcotics, differences between life stages may be more important, and recovery may be less predictable. For TKTD models, a flexible test design does not provide a fundamental obstacle: as our analysis shows, different calibration data will tend to affect precision (the confidence intervals) rather than the accuracy of parameter estimates. More specific recommendations on test design will depend on the purpose of the analysis: for the estimation of a single parameter (such as the NEC), optimal test design is different than for the link to population models (see [35]).

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4. Integrated assessment of the effects of the putative endocrine disruptor tributyltin on *Lymnaea stagnalis* using the DEBkiss model

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Abstract

Ecotoxicological research on endocrine disruption in vertebrates is rapidly progressing. Despite of high sensitivity of molluscs to endocrine disruption, a comprehensive understanding of endocrine disruption in this animal group, and in invertebrates in general, currently cannot be achieved due to the lack of standardised mechanistic tests. However, tokicokinetic-toxicodynamic (TKTD) models calibrated upon data obtained in toxicity tests with integrative (apical) endpoints may facilitate understanding of effects of endocrine disruptors (EDs). In the present study we combine experimental and TKTD modelling approaches to assess adverse effects of the putative ED tributyltin (TBT) in the great pond snail *Lymnaea stagnalis*. We performed apical toxicity tests in which embryos, sub-adult, and adult snails were exposed to TBT. Effects of TBT on survival, growth, reproduction, egg quality, and embryonic development were monitored over time. A generic TKTD model, *i.e.* DEBkiss, was successfully parameterised and calibrated based upon data obtained from two independent toxicity tests on sub-adults and adults, simultaneously. The DEBkiss analysis suggested a decrease of energy assimilation, *i.e.* a decrease of the feeding rate, as the metabolic mode of action of TBT. Estimated no-effect concentrations for TBT in terms of survival and sub-lethal effects were 1386 and 49.5 ng Sn/L, respectively. Furthermore, the model calibrated in data from toxicity tests with sub-adults and adults was used to predict embryonic development. DEBkiss did not predict embryonic development with the satisfactory level. This result may indicate that in embryos TBT affects different aspects of assimilation processes comparing to sub-adults and adults. We further discuss how the metabolic mode of action of TBT might be related to endocrine disruption. Finally, we tackle the question about the necessity of identifying effects of chemicals on the endocrine system, in the context of the risk assessment of plant protection products and biocides.

4.1. Introduction

A proper demonstration of endocrine disruption requires establishing causal links between endocrine properties (as assessed in *in vitro* studies) and adverse effects (as assessed in *in vivo* studies) [1]. Studies aiming at establishing these links usually use a bottom-up approach, as suggested by the Organisation for Economic Co-operation and Development (OECD) Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals [2]. This approach, which also forms the basis of the adverse outcome pathways approach (AOP) [3], is intended to help understanding the cascade of reactions involved in endocrine disruption. There is a number of vertebrate screens and *in vitro* and *in vivo* toxicity tests [2] that are suitable for a weight of evidence judgement on the mechanisms of endocrine disruption. Yet, not all possible modalities of endocrine disruption are at present covered by the tests. A reasonably complete suite of standardised assays for testing the effects of endocrine active substances is available for vertebrates for the estrogenic, androgenic and other steroidogenic modalities and the thyroid modality [4]. Other modalities of endocrine disruption, such as *e.g.* retinoid or peroxisome proliferator-activated receptor signalling pathways alteration and many others, are not covered yet [4]. For invertebrate species, the palette of screening and toxicity test methods is much more restricted, mainly due to limited knowledge about invertebrate endocrinology [5]. This consequently leads to a poor understanding of mechanisms of toxicity of endocrine active substances in invertebrates.

Alternatively, a top-down approach may be considered to investigate endocrine disruption: starting from the observed effects on life-history traits relevant for population sustainability (*e.g.*, survival, growth, and reproduction) to work back to the underlying mechanisms of toxicity. For example, Swain et al. [6] demonstrated that a top-down approach could guide mechanistic studies at the molecular level in order to shed light on the energetic basis of toxic effects on life-cycle traits in the nematode *Caenorabditis elegans*.

Effect models based on the theory of Dynamic Energy Budgets (DEB) [7] follow a top-down strategy: analysing life-history traits of an individual over its entire life cycle leads to hypotheses about how energy allocation to different metabolic processes is affected by the chemical. Metabolic processes in an organism are under hormonal control. Yet, the control pathways are not detailed in DEB theory as the assumptions underlying DEB theory operate at a higher level of abstraction. For example, a key assumption in DEB theory is that a constant fraction κ of the assimilated energy from food is allocated to the soma for growth and maintenance processes. The remainder is used for maturation and reproduction. It is likely that the value of κ is under hormonal regulation, which ensures the validity of assuming a constant value for κ. A disruption of the hormonal system could result in a different value of this model parameter, with consequences that can be observed from the life-history traits values over the life cycle [8]. A top-down approach like DEB can thus identify a change in κ as the most likely mechanism for a chemical stressor, but not the exact pathways leading to this change.

In DEB theory, a toxicant exerts its toxicity via one or more metabolic modes of action (mMoA, see [9]). The mMoA represents a concerted pattern of effects on life-history traits (*a.o.*, size and fecundity) over time that result from the change in one (or few) broad-scale metabolic process(es) (*e.g.*, assimilation or maintenance). The most likely mMoA can be deduced from the observed patterns of effects on life-history traits. To avoid ambiguities, we need to make a clear distinction between the mMoA in DEB theory and two other commonly used terms: the mode and the mechanism of action of a chemical. The mode of action is defined as a common set of physiological and behavioural signs that characterises an adverse biological response, while the mechanism of action is defined as the detailed understanding of the sequence of events that lead to a toxic outcome [10], as viewed in the AOP framework [3]. The mMoA is thus somewhere in between these two commonly used terms: selecting a mMoA does not require knowing the whole molecular cascade of effects as required for the 'mechanism of action', but is more mechanism-based than the 'mode of action'. The mMoA thus complements the mechanism of action, providing the link

between events at the molecular level and effects occurring at the level of the individual. Knowing the correct mMoA is essential to extrapolate effects to untested environmental conditions [11], and may be of relevance for extrapolations to the population-level effects [12].

At this moment, the same set of generic mMoAs is used to model adverse effects of all chemicals in all species [9]. It is unclear whether DEB models are generically able to describe patterns of effects of endocrine disruptors (ED) on life-cycle traits without additional physiological assumptions. It is possible that the effects of EDs can be readily described by the current models and mMoAs because the endocrine system controls the metabolic processes that underlie the mMoAs of toxicants. For instance, the standard DEB model was successfully used to model the effects of nonylphenol on the life-cycle traits of the marine polycheate *Capitella telata* without additional hypotheses about the animal's physiology or the chemical's mode of action [13]. We do not know if this finding generally applies to other species and endocrine active substances as the physiological mechanisms that underlie the different mMoAs in DEB theory are unknown.

In this study, we assess the ability of a recently developed simplified DEB model (DEBkiss) [14] to describe biological responses of the great pond snail *Lymnaea stagnalis* exposed to tributyltin (TBT). TBT has been chosen for this case-study because it is suspected to induce endocrine disruption in gonochoristic gastropods by masculinisation of females (*i.e.*, imposex, intersex, and ovo-testis development), as demonstrated in more than 260 gastropod species [15]. Previous studies in *L. stagnalis* also suggested that endocrine disruption might explain some reproductive effects observed in this species [16]. However, the exact mechanism of action of TBT in molluscs, including *L. stagnalis*, is not fully established. For TBT, several hypotheses have been proposed about the mechanisms of action for imposex development, as reviewed by Sternberg et al. [17]: steroid, neuropeptide, and a retinoid X receptor (RXR) hypotheses. It appears that those may not be the only possible mechanisms; a cross talk of some of these (*e.g.*, steroid and RXR pathways[18]) or other mechanisms (*e.g.*, peroxisome proliferator-activated receptor pathways [19]) are plausible as well.

The neuroendocrine control in invertebrates is highly variable among taxa due to the complexity and diversity of reproductive systems and life cycles [20, 21]. The presence of both sexes in an individual makes the neuroendocrine system of *L. stagnalis* distinct in comparison to that of gonochoristic species. Effects of endocrine-disrupting chemicals on *L. stagnalis* might thus be different and may occur through different pathways.

The main question we address here is whether TBT induces patterns of adverse effects that potentially indicate endocrine disruption in *L. stagnalis* (*e.g.*, non-monotonic concentration-response curves or effects at low exposure concentrations). This was assessed for a suite of endpoints related to survival, growth, reproduction, egg quality, and embryonic development. When considering the responses to TBT for this suite of endpoints, can we consider endocrine disruption as a putative mechanism of toxicity of TBT in *L. stagnalis*? Furthermore, can these effect patterns be adequately described by the generic DEBkiss model, as previously used for acetone in this species [22]? We use the results from this case study to discuss the possibilities of using a DEBkiss model for the analysis of adverse effects of putative EDs in *L. stagnalis*. We further discuss how the mMoA in DEB might be related to endocrine disruption. Finally, we will tackle the question about the necessity of identifying effects of chemicals on the endocrine system, in the context of the risk assessment of plant protection products and biocides.

4.2. Material and methods

4.2.1. Description of the DEBkiss model

In this study, we used the DEBkiss model [14] to analyse effects of TBT on life-cycle traits of *L. stagnalis*. DEBkiss falls into the category of toxicokinetic-toxicodynamic (TK-TD) models, which

describe toxicity as a process in time. It is based on the principles of Dynamic Energy Budget (DEB) theory [23] and deals with the full life cycle of an organism (including the embryonic stage). Compared to the standard DEB animal model [7], DEBkiss does not assume a division of the biomass into a reserve and a structure component, and includes a different approach for the embryonic stage (see Appendix 2). It shares however the aspects of mass balance, full life cycle, and generality (*i.e.*, species differ more in parameter values than in the structure of metabolic organisation). These model features make DEBkiss relatively easily applicable in the analysis of ecotoxicity tests data, particularly of invertebrates. Furthermore, the DEBkiss model already proved to be suitable to describe life-cycle traits of *L. stagnalis* under (toxic) stress [14, 22]. Here, we use the same DEBkiss formulation for toxic effects as applied earlier [22]; the full set of model equations can be seen in Appendix 2. However, for the present discussion it is helpful to recall some of the most important features of this model.

Before a toxicant can exert an effect, it first must enter the organism and reach the target site (toxicokinetics, TK) where it will affect a metabolic process (toxicodynamics, TD). For modelling the TK of TBT we used a scaled first-order one-compartment model, which accounts for the effects of changes in body size and losses due to reproduction. For TD, we assume that the scaled internal concentration affects one or more of the model parameters that describe metabolic processes (*e.g.*, assimilation or somatic maintenance). One of the model parameters in DEBkiss is of particular relevance for environmental risk assessment: the no-effect concentration (NEC). The NEC represents a threshold concentration below which there are no effects on the endpoint, *i.e.* on (i) survival and (ii) growth and reproduction, even after prolonged exposure.

In comparison to the DEBkiss analysis of Barsi et al. [22], we made two modifications to the model. First, a constant factor (z) was included to account for the apparent differences in bioavailability between the two partial life-cycle studies, probably caused by a difference in solvent concentration (see next section). Second, we added a Michaelis-Menten saturation on the uptake kinetics to account for the saturating effect patterns observed at higher exposures (especially on survival) (see [24, 25]). This saturation was applied as a modification of the external concentration (c):

$$c^* = \frac{c \times c_K}{c + c_K}$$

where c_K is the half-saturation concentration.

4.2.2. Test animals and test chemical

Snails (*Lymnaea stagnalis*, RENILYS® strain) were cultured at the INRA Experimental Unit of Aquatic Ecology and Ecotoxicology (Rennes, France), under conditions previously described [26]. Culture and test medium was dechlorinated charcoal-filtered tap water. The test medium was renewed twice a week to maintain its physico-chemical properties and to avoid fouling of test vessels. The vessels were additionally cleaned with soft paper tissue on each renewal. Animals were fed *ad libitum* with rinsed fresh organic lettuce. Photoperiod was 14:10 hours light:dark. Prior to exposure, all snails passed a two-day acclimation period in test beakers, which ensured that they had recovered from any stress due to transfer from the culture.

Tributyltin chloride (CAS number 1461-22-9, Aldrich®, purity > 96%) was dissolved in acetone (CAS number 67-64-1, Sigma-Aldrich®, purity ≥ 99.9% (CHROMASOLV®)) to prepare stock solutions. TBT was incorporated in the test medium by adding the appropriate volume of stock solution to each test beaker, using a micropipette, after removal of an equivalent volume of test medium. Nominal concentration of the carrier solvent at contaminations of the test medium was the same in all treatments and was 20 µL/L in sub-adult and embryo development tests and 2 µL/L in the test with adult snails (see test design). These concentrations should not lead to adverse effects, as the NEC for growth and reproduction for acetone has been estimated as 880 µL/L [22]. The

selected acetone concentrations are also in accordance with the recommendations for the use of carrier solvents in toxicity tests made by the OECD and Hutchinson et al. [27, 28].

4.2.3. Test design

The data for the model calibration were obtained from two independent partial life-cycle toxicity tests in which sub-adult and adult snails were exposed to a range of TBT concentrations. Sub-adult snails became adults (onset of egg production) during the tests, but we continue referring to them as sub-adults in the present paper. The two partial life-cycle toxicity tests were performed at a different time and for a different purpose, and therefore differed in their design. The data from both tests were used to calibrate the DEBkiss model (more about benefits of using flexible tests design to calibrate TK-TD models is provided in Barsi et al. [22]). Furthermore, we assessed the quality of eggs collected from the sub-adult test. Finally, to assess effects of TBT on embryonic development, we performed a partial life-cycle toxicity test with embryos exposed to a range of TBT concentrations.

Partial life-cycle test with sub-adult snails. Sub-adult snails were sampled from the laboratory culture and placed individually into glass beakers filled with 200 mL of test medium. The temperature of the test medium was 22.2 ± 1.3°C. Mean initial shell length and age of snails after the acclimation period was 20.1 ± 1.2 mm and 144 d, respectively.

Sub-adult snails (four per treatment) were exposed in isolation to 10 nominal TBT concentrations ranging from 11 to 2743 ng Sn/L during 35 d. This concentration range was selected based upon earlier results of toxicity tests with TBT in *L. stagnalis* [16, 29, 30] and different gastropod species [31-33]. With a wide concentration range, we aimed at detecting effects of both low and high concentrations of TBT, which is relevant for endocrine disruption [4]. The multiplication factor between concentrations was 2.2, as recommended by the OECD [34], except at three highest concentrations where this factor was set to 1.3 in order to increase the test resolution where high mortality is to be expected. Twenty-two isolated, non-exposed snails served as a control group. More animals were used than in the exposure groups to obtain precise estimates for the basic metabolic parameters of DEBkiss. Of this number, four snails were sacrificed at d 0 and 21 to measure the dry weight of the soft body tissue, four snails served as a solvent control, and the remaining 10 snails served as a clean water control group. A t-test confirmed no significant difference between the clean water and the solvent control groups ($p > 0.05$), and therefore data from these two groups were pooled.

Survival was monitored daily. Dead snails were removed from the test beakers and stored at -20° C. Body size was assessed from the shell length, measured once a week using a digital calliper, and from dry weight of soft body tissue, determined only once at the end of the test (or when an individual died). To obtain dry weights of the soft body tissue, snails were separated from their shells and freeze-dried individually during 48 hours. To assess effects on reproduction, egg clutches were removed daily from the walls of test beakers with a sharp-edged spoon. Eggs were then counted under a stereo-microscope. Furthermore, we quantified reproductive investment per snail through the cumulative dry weight of egg clutches. Egg clutches were placed individually into plastic micro-tubes and freeze-dried during 24 hours, and weighed afterwards. The dry weight per egg, which is a parameter in the DEBkiss model, was determined from all clutches collected in the control. To decrease the potential impact of isolation on the snails' reproductive performance, we allowed them to mate regularly during the tests. Snails were coupled (always using the same couple) twice a week for 8 hours, which sufficed to allow snails to mate [35]. Finally, we assessed egg quality for all egg clutches collected during the test. The frequency of three types of abnormalities was determined: polyembryonic eggs, unfertilized eggs, and eggs with atrophied albumen (see [16] for the explanation of the types of abnormalities).

Partial life-cycle test with adult snails. The test on adult snails was designed based upon recommendations given by the OECD [34], and was performed as described by Giusti et al. [16].

Six replicates (each with five snails in 1-L glass beakers) were used per treatment (four test concentrations, a water control, and a solvent control). Snails were exposed to four nominal TBT concentrations from 133 to 1065 ng Sn/L during 28 d. The temperature of the test medium was 19.5 ± 0.7°C.

Survival was monitored by counting and removing dead snails from beakers twice a week. Individual shell length was measured once a week using a digital calliper. Dry weight of soft bodies was obtained from 10 randomly selected snails per treatment at the end of the test and was expressed as a mean value. Effects on reproduction were estimated by monitoring the cumulated number of eggs per snail twice a week. Clutches were collected and eggs were counted under a stereo-microscope. Dry weight of 10 randomly selected clutches, collected from control snails at d 7, 14 and 25, was measured in order to estimate the mean dry weight of a single egg.

Partial-life cycle test with embryos. To obtain data on embryonic development under TBT stress, we conducted a toxicity test based on the method of Bandow et al. [36]. Eggs were extracted from jelly-masses laid within a 24-hour period, and randomly placed into a 12-well plastic plate (one egg per well) per treatment. Each well was previously filled with 10 mL of contaminated test medium. Five exposure treatments were set with TBT nominal concentrations from 11 to 1995 ng Sn/L. The test lasted 21 d, and was performed at a temperature of 22.4 ± 1.1°C with a photoperiod of 14:10 hours light:dark. The following endpoints were monitored: hatching success after 21 d (considered as a proxy for survival in our case), time to hatch, and shell length of hatchlings. A more detailed description of the test design and test conditions is available in Chapter 3.

4.2.4. Model calibration

First, DEBkiss was fitted simultaneously to all data on survival, growth, and reproduction of control snails in the sub-adult and adult partial life-cycle tests. We accounted for the temperature difference in the two toxicity tests via an Arrhenius temperature correction. This correction requires the value of the Arrhenius temperature (T_A), for which we used the value of 8000 K [37]. The same temperature correction factor is applied to all rate constants (*i.e.*, all parameters with a dimension that includes "per time"). The basic metabolic parameters from the analysis of the control snails were fixed in the subsequent analysis of the exposed snails. This is defensible given the larger number of control snails, and simplifies the analyses considerably. The model was implemented in Matlab 2010b, and parameter values were estimated by maximising the overall likelihood function [38] (using square-root transformation of data for the continuous endpoints). The 95% confidence intervals on parameter estimates were generated by profiling the likelihood function [38]. The mMoA was selected based upon the best fit to all available data for exposed sub-adult and adult snails simultaneously. The model parameters are presented in Table 4.1.

4.2.5. Statistical analysis of the data on egg quality and on the embryonic development

Effects of TBT on the frequency of egg abnormalities (eggs laid by exposed parents) and on the embryonic development (eggs laid by unexposed parents) were assessed using standard statistical analysis procedures as described in OECD [39], with a significance level of 0.05. Note that these data were not used to calibrate the DEBkiss model.

Analysis of the egg quality was based on the total number of collected clutches. Statistical differences in the frequency of abnormal eggs were assessed using the Mann-Whitney test with Bonferroni-Holm adjusted *p* value. The analysis was performed in SPSS (v. 20) software (IBM SPSS, Armonk, NY).

Differences in the hatching success among treatments were assessed with the Cochran-Armitage trend test using JMP® (v. 10) software (SAS Institute Inc., Cary, NC). Data on hatching time and hatchling size were analysed with the Jonckheere-Terpstra test, using the SPSS software.

Table 4.1. Parameter values as estimated based on best fit to test data with *L. stagnalis.*

Metabolic model parameters	**Symbol**	**Value**	**Unit**
Specific maximum assimilation rate	J_{Am} [a]	0.159 (CI: 0.141-0.181)	mg/mm^2 d
Specific maintenance rate	J_M^v	$8.34 \cdot 10^{-3}$ (CI: $6.90 \cdot 10^{-3}$-$9.85 \cdot 10^{-3}$)	mg/mm^3 d
Allocation fraction to soma	κ	0.645 (CI: 0.628-0.661)	-
Shell length at maturation	L_{wp}	25.1 (CI: 24.8-25.4)	mm
Blank hazard rate	h_0	$5.21 \cdot 10^{-20}$ (CI: 0-$8.51 \cdot 10^{-4}$)	1/d
Shape correction coefficient	δ_M	0.369 (SE: $4.7 \cdot 10^{-2}$) [a]	-
Dry-weight density of structure	d_V	0.105[a] (SE: $1.6 \cdot 10^{-3}$) [a]	mg/mm^3
Dry weight of a freshly-laid egg	W_{B0}	0.13[b] (SE: $3 \cdot 10^{-3}$) sub-adults [b] 0.18[b] (SE : $4 \cdot 10^{-3}$) adults [b]	mg mg
Yield of egg buffer on assimilates	y_{BA}	0.95 [c]	mg/mg
Yield of structure on assimilates	y_{VA}	0.8 [c]	mg/mg
Scaled functional response	f	1 [c]	-
Arrhenius temperature	T_A	8000 [d]	K
Toxicological model parameters			
Elimination rate constant	k_e	$4.64 \cdot 10^{-2}$ (CI: $3.79 \cdot 10^{-2}$-$5.46 \cdot 10^{-2}$)	1/d
NEC for survival	c_{0s}	1390 (CI: 961-1910)	ng/L
Killing rate	b	$2.96 \cdot 10^{-3}$ (CI: $8.83 \cdot 10^{-4}$-$9.29 \cdot 10^{-3}$)	L/mL d
NEC for metabolic effects	c_0	49.5 (CI: 42.6-55.7)	ng/L
Tolerance concentration	c_T	202 (CI: 158-241)	ng/L
Yield of assimilates on structure	y_{AV}	0.614 (CI: 0.468-0.824)	mg/mg
Half-saturation concentration	c_K	2080 (CI: 1160-5230)	ng/L
Bioavailability factor (test with adults)	z	0.585 (CI: 0.540-0.633)	-

[a] Parameter values are not calibrated but fixed to the suggested values [22].
[b] Parameter values were derived from the two toxicity tests with TBT.
[c] Parameter values are not calibrated but fixed to the suggested values [14].
[d] The parameter value was not calibrated but fixed to the suggested value [37].
CI = 95% confidence interval; SE = standard error; NEC = no-effect concentration.

4.3. Results and discussion

4.3.1. Effects of TBT on survival, growth and fecundity

TBT affected growth and reproduction of sub-adult and adult snails, and survival of sub-adult snails only (Figure 4.1). Sub-adults showed much more effect on growth and reproduction than adults at similar exposure concentrations. This is unexpected based upon the DEB model assumptions: we assume that the intrinsic sensitivity, *i.e.* the relationship between the internal concentration and the affected metabolic process (*sensu* Pieters et al. [40]), does not depend on the developmental stage of the individual. This assumption generally applies in a number of species and toxicants, and was confirmed in *L. stagnalis* for acetone in [22] and diquat [41]. We also assume that there no a substantial difference in the complexity of the neuroendocrine system between sub-adults and adults. For TBT, we hypothesise that the difference in the effect magnitude between the two tests in this study represents a difference in bioavailability of TBT, as the concentration of the carrier solvent was 10 times lower in the adult test. Therefore, in the DEBkiss model, we fitted a correction factor *z* (Table 4.1) on the exposure concentration from the study with adult snails. A factor of less than 2 was sufficient to explain both tests with the same set of toxicological parameters.

To adequately describe the effects of TBT at high exposure concentrations (mainly on survival) we added saturation on the uptake kinetics. This allows capturing the observed pattern that

increasing concentrations produced less than the expected increase in effect. Saturation of TBT concentrations in aquatic molluscs has already been observed, *e.g.* in the clam species *Venerupis decussate* [24]. Furthermore, in other DEB-based studies (*e.g.*, [25]), a saturation of effects has been addressed in the same manner as in our study. With these two model adaptations, the complete set of data from the two partial life-cycle toxicity tests was well described by the model (Figure 4.1).

The DEBkiss model allowed estimating the values of NECs of TBT in snails. The NEC for survival and the NEC for growth and reproduction (cumulative fecundity and cumulative dry weight of clutches) were 1386 (95% CI: 961-1910) and 49.5 (95% CI: 42.6-55.7) ng Sn/L, respectively.

The value of NEC for survival is in a good agreement with previous findings where TBT did not affect survival of snails after 21-d exposure to 1065 ng Sn/L [16], after 56-d exposure to 180 ng Sn/L (unpublished data from a round-robin test), and after 84-d exposure to 100 ng Sn/L [29]. However, Segner et al. [42] reported 290 ng Sn/L as a 21-d LC_{50} (a concentration that induces mortality of 50% of animals) for 40 d old juveniles and 80 d old adults of *L. stagnalis*, although no experimental description was provided to scrutinize the test results. Furthermore, in a 170-d toxicity test conducted by Leung et al. [30], TBT oxide significantly reduced survival of *L. stagnalis* at 3.5 times lower concentration than our NEC for survival. Unlike our study, Leung et al. exposed snails life-long to a different formulation of TBT, starting already from egg clutches. This highlights a possibility that there might be a difference in bioavailability or toxicity between the two chemicals and/or that the exposure during the early phases of the life-cycle affects sensitivity later in life.

The estimated NEC for growth and reproduction of 49.5 ng Sn/L is lower than the NOEC (no-observed effect concentration) of 220 ng Sn/L for shell size and of 100 ng Sn/L for cumulative fecundity, as reported in Giusti et al. [16] and whose study included only adult stage. As the NOEC is generally associated with some level of effect [43], results from the present study are consistent with our previous findings. In other studies, NOEC values for cumulative fecundity of 10 [29] and 4 ng Sn/L [30] were obtained, which is lower than the NEC. Overall, the NEC for sub-lethal effects is consistent with results from the available literature.

4.3.3. Effects of TBT on egg quality

TBT did not affect the frequency of any of the three monitored types of egg abnormalities. This is in contrast with the study of Giusti et al. [16] who observed a significant effect of TBT on the frequency of egg abnormalities at a similar concentration range, polyembryony being the most frequently observed type of the abnormalities. The authors concluded that frequency of polyembryonic eggs was the most sensitive endpoint in the study and suggested endocrine disruption as a putative mechanism of TBT in *L. stagnalis*. The difference between this and our study may be driven by a difference in the duration of the reproduction phase of snails at the moment of sampling; we assessed abnormalities on eggs from snails who just entered the reproduction phase, while in the other study snails were already reproductively active for a much longer time. The carrier solvent might as well have interfered with responses to TBT as some carrier solvents, including acetone, can modulate effects of chemicals [44]. It has been shown that acetone can increase a frequency of polyembryonic eggs, although this only happens at concentrations of at least a factor of 10 higher than applied in these studies [45].

4.3.4. Effects of TBT on embryonic development

TBT significantly impaired hatching success (survival) at the concentrations of 907 and 1995 ng Sn/L (Figure 4.2), which is comparable with the findings of Leung et al. [30] for TBT-oxide. Because parents were not exposed to TBT, it is likely that the compound is indeed taken up into the eggs. Effects of TBT on hatching success occur at nearly the same concentrations which are causing mortality in sub-adults (see Figure 4.1), suggesting a similar intrinsic sensitivity of embryos and sub-adult snails. Note that solvent concentrations differed between tests with sub-adults and adults,

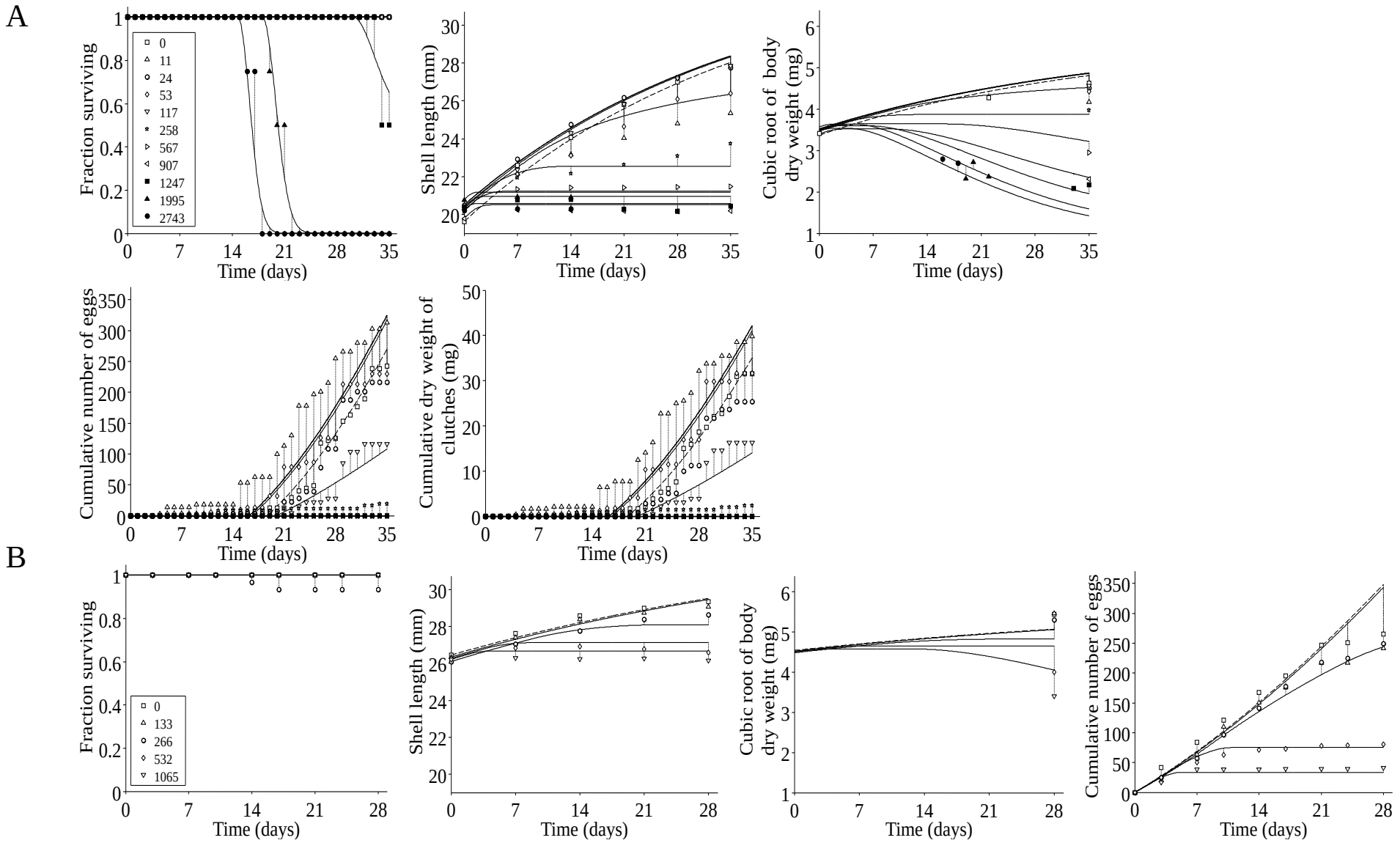

Figure 4.1. Effects of TBT (ng Sn/L) on survival, shell length, dry weight of the soft body, and cumulative number of eggs of sub-adult (panel A) and adult (panel B) snails. Cumulative dry weight of clutches was additionally monitored in the test with sub-adults. Symbols represent the observed average values of snail responses. Broken lines are model fits to control data while full lines represent model fits to the data on exposed snails. In the test with adults, note a greater deviation of model fits to the data on cumulative number of eggs between d 21 and 28 due to a temperature drop in the laboratory room. Data on reproduction from this period thus were not used for the model calibration.

and as the model accommodates for this difference, we may conclude that the intrinsic sensitivity is similar for all the three tested age classes. There were no clear effects of TBT on time to hatch and on hatchling size (Figure 4.2). The concentration at which hatching success was significantly affected in this study is higher than the 123 ng Sn/L as observed by Bandow et al. [36]. Differences in the test design might explain this discrepancy, *e.g.* differences between test media or the fact that Bandow et al. did not use a solvent.

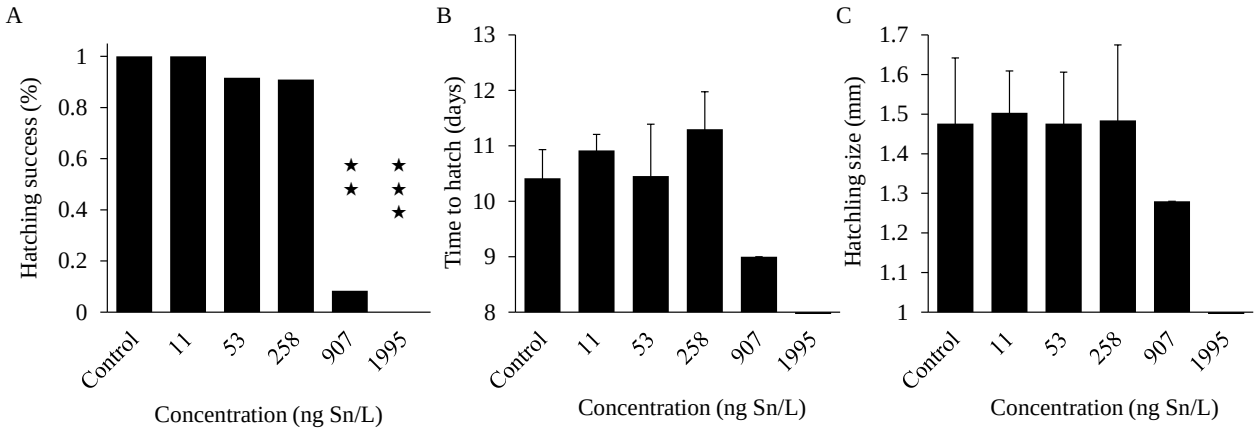

Figure 4.2. Embryo exposure test. Eggs were extracted from their jelly-mass and exposed directly to TBT (ng Sn/L). Presented are effects of TBT on A) hatching success, B) time to hatch, and C) hatchling size. Error bars represent the standard error. Two and three asterisks correspond to $p < 0.01$ and $p < 0.001$, respectively.

4.3.5. mMoA of TBT in L. stagnalis and links with the putative mode of action

The overall pattern of effects on growth and reproduction of snails indicates that TBT affects processes related to either energy assimilation or maintenance. Our DEBkiss analysis strongly suggests that TBT decreases assimilation of energy in *L. stagnalis* as this mMoA provides the best model fit to the data on survival, growth, and reproduction.

In our study, a decrease in energy assimilation can result from either a decrease in the assimilation efficiency or a decrease in the feeding rate, as these two options will be indistinguishable from their consequences on growth and reproduction. However, the visual evidence that food consumption decreased in TBT treatments (Figure 4.3), which confirms the finding of Giusti et al. [16], supports the hypothesis that a decrease of the feeding rate has caused the decreased energy assimilation in the snails.

In DEBkiss, effects on assimilation as observed in sub-adults and adults are also expected to affect hatching time and hatchling size of the embryos directly exposed to TBT (embryos originated from non-exposed snails from the laboratory culture). Indeed, the model assumes that the embryo assimilates the buffer of reserve material in the egg (endogenous feeding). These theoretical expectations were experimentally confirmed for the effects of acetone in *L. stagnalis* [29]. Yet, they were not confirmed in the present study with TBT. The lack of effects on hatching time and hatchling size of embryos, at concentrations where the sub-adult and adult snails show clear effects on growth and reproduction, indicates that TBT affects an aspect of the feeding process that is specific for exogenous feeding of the snails on lettuce.

Effect of a toxicant on energy assimilation is not a mMoA that is specific for endocrine disruption. Effects on assimilation can be induced by a number of non-endocrine-active substances from diverse chemical groups, *e.g.* the narcotic acetone [22] and the herbicide diquat in *L. stagnalis* [41], and uranium in *Daphnia magna* [46].

Our test design does not allow obtaining information relevant for defining the molecular mechanism of how TBT affects feeding rate of snails. It may well be that TBT affects functioning of the digestive gland, which might consequently influence the feeding rate. Snails exposed to

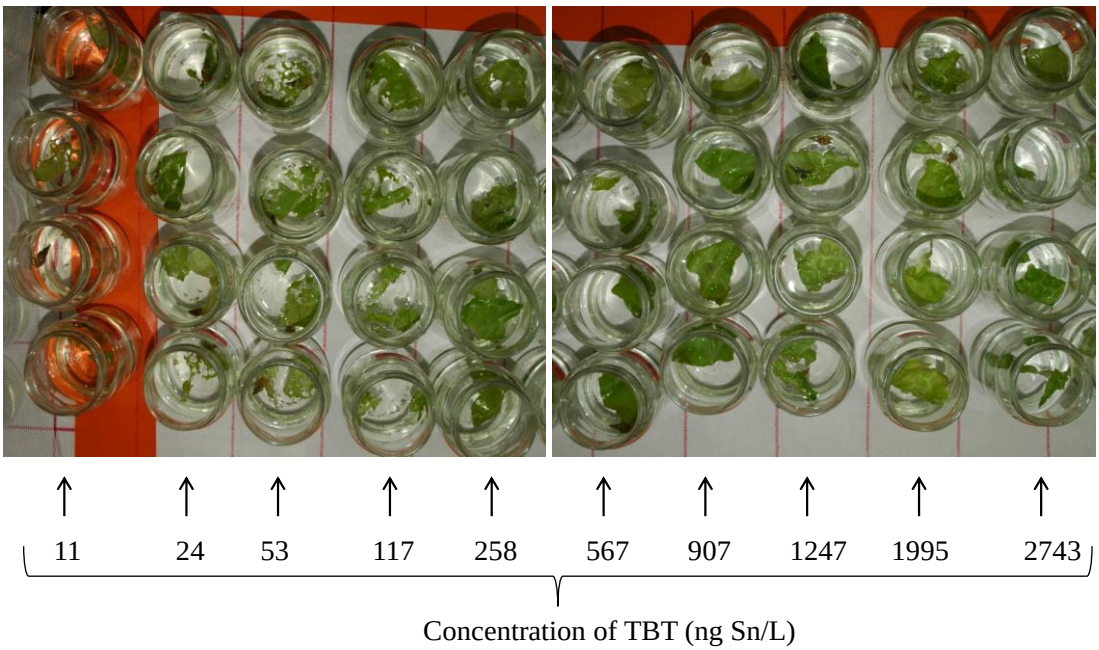

Figure 4.3. Decreased food consumption of sub-adult snails in TBT treatments. Lettuce was consumed in low-concentration treatments (beakers on the left side on the photo), while in medium- and high-concentration treatments its consumption was limited (beakers in the middle and on the right side on the photo). The observation was made after 24 hours of the moment when the lettuce was provided. The photos are taken at d 7 of the test.

TBT concentrations of 907 ng Sn/L and higher had a considerably decreased colour intensity of the body part where the digestive gland is located, as seen through the shells (Figure 4.4), which suggest alterations of the functions of this gland. It can also be seen that there is a lack of gut contents in animals at higher exposures (Figure 4.4). Further, based upon the results of a histopathological investigation, Segner et al. [42] reported a capability of TBT to disturb the development of secretory cells of the digestive gland at 131 ng Sn/L in 40 d old juveniles and necrosis of digestive gland at 1310 ng Sn/L in 80 d old adults. Indeed, the digestive gland of *L. stagnalis* has a major role in both detoxification processes of the organism and in the secretion of digestive enzymes [47]. Therefore, it is plausible that the observed decrease of feeding rate was a consequence of inability of snails to digest food. Food digestion further can be impacted due to overt toxicity on the digestive gland or due to its increased detoxification activity. Assuming that TBT directly alters the functioning of the digestive gland, this chemical would not be considered as an endocrine disruptor in *L. stagnalis*.

Alternatively, feeding rate in snails might be affected via the neuroendocrine pathways. Currently, the most studied hypothesis is that RXR signalling pathways are involved in imposex development of prosobranchs, although the exact role of the RXR in hormonal regulation of snails is still unknown [17]. TBT binds the RXR of prosobranch snails with a similar affinity as its natural ligand (retinoic acid) and also induces transcriptional activity [48, 49]. In adults of *L. stagnalis* the RXR has been identified in neurons, the digestive gland, haemocytes, and in embryos (as a whole organism), which suggests a role of this receptor in neuroendocrine communication, detoxification, and embryogenesis [47, 50]. Like in prosobranch snails, retinoic acid is the ligand of the RXR of *L. stagnalis* [50], so it is likely that it binds TBT as well. Furthermore, TBT greatly accumulates in neurons of some aquatic snail species such as the mud snail *Ilyanassa obsoleta* [51]. It has been argued that TBT causes abnormal release of a neuropeptide hormone APGWamide that induces imposex in this species [51]. Thus, the RXR might mediate the release of APGWamide in *I. obsoleta* exposed to TBT.

In *L. stagnalis* a few molluscan insulin-related peptides (MIPs) exist, which are secreted in light green cells of the central nervous system and regulate metabolism and growth [52]. APGWamide affects light green cells of cerebral ganglia so that the secretion of some of MIPs may be inhibited

[53], but it cannot be excluded that the RXR might directly inhibit the release of MIPs from light green cells. This might explain the observed effects of TBT on growth and consequently on reproduction. Yet, it does not explain the observed decrease in the feeding rate. It might be that other mechanism alone or in a conjunction with the previously mentioned is at play. For example, neuropeptides FRFamide and MIP VII are present in neurons of the buccal ganglia in *L. stagnalis*, suggesting their role in feeding process. In the marine snail *Aplysia californica* FRFamide inhibits contractions of the buccal muscle and decreases feeding ability, which has also been suggested for MIP VII [54]. Therefore, in *L. stagnalis* exposed to TBT the RXR might induce release of these neuropeptides in the buccal ganglia, which might explain effects on decreased feeding rate observed in our experiments.

Feeding is a complex activity, which in *L. stagnalis* employs about 100 neurones that contribute to distributed network functions in which neurons share their roles and modulate their activities simultaneously [55]. Hence, it is probable that a several regions of the central nervous system and/or several type of neurons controls the feeding behaviour. Consequently, we may speculate that several types of neurons or network functions could be a target of TBT. Alternatively, TBT might initiate a down-regulation of the digestive gland functions via the RXR or other signalling pathways, which then might also consequently affect feeding ability of snails.

Overall, more research is needed to elucidate the mechanisms underlying the effects of TBT in *L. stagnalis.* Histopatological studies of different organs or elucidation of biochemical pathways following exposure of snails to TBT can be a step forward in this respect. It would be interesting to get better insight on what exact damage TBT causes on the digestive gland, to check the distribution of TBT in tissues, organs and egg masses while still in the body, to investigate effects of TBT on the secretion and functioning of some potentially affected neuropeptides, or to conduct studies on binding of TBT to suspected receptors. This would help to better understand the links between the mMoA of TBT and its molecular mechanism of action. Despite the uncertainties about the molecular mechanism of TBT toxicity, DEBkiss offers a coherent, biologically relevant, and theoretically consistent explanation of the effects of TBT on life-cycle traits of *L. stagnalis.* In this respect, the DEBkiss model is useful to interpret the effects of TBT on apical endpoints in snails.

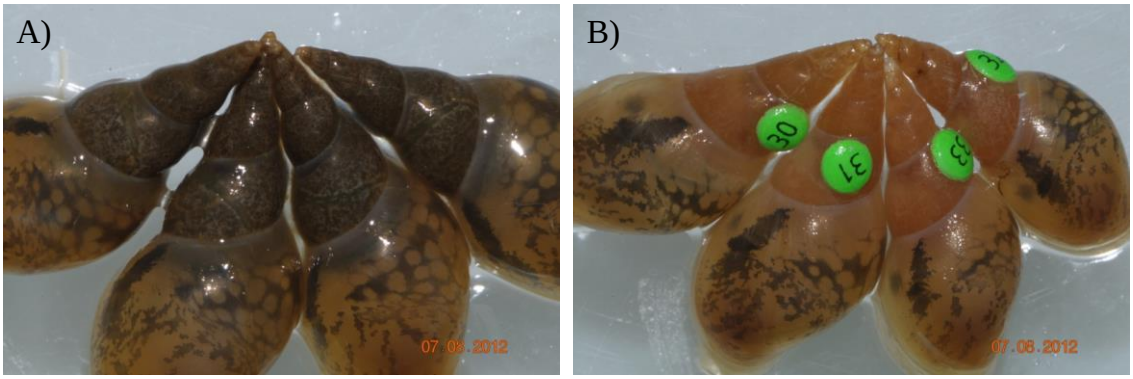

Figure 4.4. Difference in colour intensity between A) control and B) exposed (2743 ng Sn/L) sub-adult snails. This observation suggests alterations of the digestive gland functioning. The photos were taken at the d 7 of the test.

4.3.6. Endocrine disruption in aquatic invertebrates and its relevance for environmental risk assessment

In standard chronic toxicity tests for aquatic invertebrates, the core endpoints are growth, reproduction, and survival. The reason to focus on these endpoints is that they are relevant for population viability. It is, however, doubtful whether specific effects of endocrine disruptors can be identified from these tests as they are not intended for that purpose. For example, an effect on the quality or quantity of sperm cells or on behaviour might easily go unnoticed as these responses are not monitored and do not necessarily have consequences for fecundity. This issue is not specific to

endocrine disruption; many chemicals have clear effects that would be missed in standard toxicity tests. As an example, some toxicants affect the size of the offspring in *Daphnia magna* reproduction tests (see *e.g.*, [56-58]). This would be missed in a standard analysis, as the focus lies on the number of offspring. However, offspring size is obviously of crucial importance to population dynamics, and hence relevant for the risk assessment of these compounds. In this respect, it is important to know which individual-level effects are actually relevant to population responses, and to use corresponding biological responses as additional endpoints for environmental risk assessment of aquatic invertebrates. As the work on endocrine disruptors forces us to consider more subtle endpoints, rather than the total number of offspring, we may also consider to incorporate these endpoints into the standard test protocols. In short, the question is not so much whether a chemical has a specific mechanism of action (such as endocrine disruption), but rather whether we are monitoring the relevant life-cycle characteristics in our standard tests.

At this moment, it remains unclear whether existing DEB-based models could deal with the specific effects of EDs. It must be stressed that DEB models deal with effects as a consequence of a change in acquisition or allocation of mass and energy. Therefore, a relevant change in *e.g.* sperm quality or mating behaviour might easily occur without a change in energy allocation. Such effects are not covered by DEB theory and would require additional modules.

In a DEBkiss analysis, effects are directly linked to changes of large-scale metabolic processes, thus ignoring the biochemical mechanisms underlying this link. This crude linkage, however, is able to provide an explanation for the combined effects of toxicants on all life-cycle traits from the exposure scenario of the individual. Nevertheless, knowledge about mechanisms of toxicity can help to formulate a TKTD model (see *e.g.*, [59]), to derive the most applicable mMoA, to focus test design on the relevant endpoints, and, in conjunction with population models (see [12, 60]), to improve the extrapolation to the field situation. In this way, DEB-based analyses and molecular/histological studies should be seen as complementary rather than exclusive alternatives.

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5. Effects of oestrogenic chemicals in snails: a case study for alkylphenols in the great pond snails *Lymnaea stagnalis*

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Abstract

In prosobranch snails, oestrogenic chemicals induce stimulatory effects on reproduction which is often linked to endocrine disruption. Information on the effects of oestrogenic chemicals in the pulmonate snail *Lymnaea stagnalis*, which is a candidate species for the OECD toxicity tests standardisation, is scarce. To obtain more data on oestrogenic effects of chemicals in this species, we performed a suite of partial life-cycle toxicity tests. Sub-adults and adults of *L. stagnalis* were exposed to alkylphenols (4-nonylphenol, 4-n-nonylphenol, and 4-tert-octylphenol) and the synthetic steroid 17α-ethynyloestradiol (EE2). Chemical effects on survival and a number of sub-lethal endpoints were monitored. Additionally, eggs were extracted from their jelly-masses and directly exposed to EE2. In all the tests, chemicals either did not affect endpoints or had negative effects only at very high, environmentally unrealistic, concentrations. In this study we discuss hypotheses for the apparent absence of the effects of oestrogenic chemicals and its relevance for environmental risk assessment of endocrine disruptors.

5.1. Introduction

Aquatic environments are often recipients of oestrogenic chemicals originating from sewage and industrial discharges, agricultural practices and livestock waste [1]. Oestrogenic chemicals are present in natural freshwaters at concentrations as low as a few ng/L. Seemingly negligible, these low concentrations are enough to induce adverse effects on wildlife such as *e.g.* feminization of fish species [2]. The feminizing effect is not typical for fishes only, but occurs in many other vertebrates, as the structure of the vertebrate oestrogen receptor mediating the effects is highly conserved across the phylum [3]. Many invertebrate species possess the oestrogen receptor (ER) as well, or its analogue, and thus similar effects as observed in vertebrates may be evidenced. However, because invertebrates form a much more diverse group (it comprises more than 30 phyla) compared to vertebrates, some deviations from the effects of oestrogenic chemicals may occur due to differences in the structure and physiological role of their ER [4].

Because molluscs have been recognized as a sensitive phylum to a number of chemicals, including the oestrogenic ones [5], the Organisation for Economic Co-operation and Development (OECD) is currently putting efforts in the development of mollusc reproduction toxicity tests [6]. Several candidate species have been proposed for the testing. Among them are the freshwater gastropods *Potamopyrgus antipodarum* and *Lymnaea stagnalis*. In aquatic gastropods, a typical adverse effect of oestrogenic chemicals is stimulation of egg or embryo production [5]. Within aquatic gastropods, the responses to oestrogenic chemicals may differ. For instance, in prosobranchs (*e.g.*, the parthenogenetic species *P. antipodarum* and the gonochorists *Marisa cornuarietis, Nucella lapillus,* and *Nassarius reticulates*) oestrogenic chemicals may induce the adverse effect at environmentally relevant concentrations [7-9]. On the other hand, the typical oestrogenic chemical for prosobranchs, 17α-ethynyloestradiol (EE2), causes no effects on the reproductive output in some pulmonates, *e.g.* the hermaphroditic species *Radix balthica* and *Physa pomilia*, even when tested at very high concentrations [10-12]. Based upon the results of these studies, we wonder if oestrogenic chemicals induce adversity in other pulmonate hermaphrodites such as *L. stagnalis*.

One of the most abundant families of oestrogenic chemicals found in freshwaters are alkylphenols. Alkylphenols and their ethoxylates are used as non-ionic surfactants in a variety of domestic and industrial applications, pharmaceutical and pesticide formulations, and textile and paper pulp production processes [13]. Because of their widespread distribution in the aquatic environment, alkylphenols exert adverse, oestrogenic effects on fish [2] and stimulate embryo production [14] or induce the syndrome called “superfemales” [8] in some prosobranch snails. Data on effects of alkylphenols and other oestrogenic chemicals on pulmonate snails are scarce. A few studies exist for *L. stagnalis*. According to these studies, adults are not particularly sensitive to 4-nonylphenol [15], while exposure of egg masses may cause an increase of embryo mortality, a delay in embryonic development and hatching [16]. Although the presence of the ER has been confirmed for most of the abovementioned molluscs [11, 17-19], it is not sure that the ER is involved in the response of exposed animals to these chemicals. Furthermore, it is not clear why the effects of the same oestrogenic chemicals differ when comparing prosobranch and pulmonate gastropods. Therefore, it is of great relevance to deeper explore effects and mechanism of toxicity of alkylphenols and other oestrogenic chemicals on *L. stagnalis*.

In this context, we investigated whether exposure to typical oestrogenic chemicals might induce adverse effects in *L. stagnalis*, as observed on apical endpoints in toxicity tests. Therefore, we implemented several partial life-cycle tests with adults, sub-adults, and embryos of *L. stagnalis.* The response of the endpoints related to survival, development, growth, and reproduction were measured. We studied effects of a suite of alkylphenols with different affinities to the vertebrate ER: 4-nonylphenol (mixture of branched isomers), 4-n-nonylphenol, and 4-tert-octylphenol. Based on the fact that in vertebrates steroid hormones and alkylphenols generate effects through binding to the ER, the use of natural or synthetic steroid hormones as a positive control in mollusc toxicity

tests for oestrogenic chemicals has been strongly recommended [5, 6, 20, 21]. Therefore, we also investigated the responses of the great pond snail to the synthetic hormone EE2.

5.2. Material and methods

5.2.1. Test animals and test chemicals

We used the great pond snail *L. stagnalis* as the test animal. Details about the culture conditions are provided in Chapter 2. Snails were exposed to the following chemicals: (i) a complex mixture of branched-chain nonylphenol isomers, *i.e.* 4-nonylphenol (NP, CAS number 84852-15-3, Aldrich®, technical grade), (ii) the nonylphenol isomer with a linear chain, *i.e.* 4-n-nonylphenol (4nNP, CAS number 104-40-5, Fluka®, analytical grade (PESTANAL®)), (iii) 4-tert-octylphenol (OP, CAS number 140-66-9, Aldrich®, purity ≥ 97%), and (iv) 17α-ethynyloestradiol (EE2, CAS number 57-63-6, Sigma®, purity ≥ 98%). In comparison with the natural steroid hormone oestradiol, the alkylphenols NP, 4nNP, and OP are considered to be weak oestrogenic chemicals. In *in vitro* tests, alkylphenols have shown 10^3 to 10^7 fold less oestrogenic potency relative to oestradiol. This potency tends to decrease from OP to 4n-NP [22-24]. Furthermore, *in vivo* tests have demonstrated no or little toxicity of alkylphenols in some mollusc species (*e.g.*, [14, 25]). In *in vitro* tests EE2 and oestradiol generally do not significantly differ in their oestrogenic potency, while *in vivo* fish tests demonstrate that the synthetic hormone is more toxic than the natural one [26], and more stable in water. These properties of EE2 make the chemical appropriate for the use as a positive control when assessing toxicity of oestrogenic chemicals in semi-static toxicity tests.

Stock solution of chemicals were prepared in acetone (CAS number 67-64-1, Sigma-Aldrich®, purity ≥ 99.9% (CHROMASOLV®)), which was used as a carrier solvent, and stored in a refrigerator at +4° C in dark. New stock solutions were prepared every week and every other week in the 28-d and 56-d testes with adult snails (see "Test design and endpoints"), respectively, and after three weeks in the 35-d test with sub-adults. For the 21-d test with embryos, we used the same stock solution of EE2 as for the 35-d test. Concentrations of stock solutions were chosen to ensure addition of the same amount of solvent among treatments (*i.e.*, 100 µL/L, except for the water control) as recommended by the OECD [27]. Nominal concentrations of the alkylphenols and EE2 and their multiplication factors were different in each test (Table 5.1). They were chosen based upon literature data on *L. stagnalis* (*e.g.*, [15]) and other aquatic gastropod species (*e.g.*, [8, 10, 14]).

Table 5.1. Partial life-cycle tests performed on *L. stagnalis*: nominal concentrations of toxicants and applied multiplication factors between the concentrations.

	Partial life-cycle tests			
	28-d test with adults	56-d test with adults	35-d test with sub-adults	21-d test with exposed offspring
Treatments (nominal concentrations in µg/L)				
NP	Not tested	0.1-500	0.05-1000	Not tested
4nNP	0.0001-10	Not tested	0.05-1000	Not tested
OP	Not tested	Not tested	0.05-1000	Not tested
EE2	0.0001-10	0.01-50	0.01-80	0.05-80
Multiplication factor between concentrations				
	10	5.5	Not defined	Not defined

5.2.2. Test design and test endpoints

A suite of partial life-cycle toxicity tests with *L. stagnalis* was performed to assess the effects of the selected chemicals on embryos, sub-adult, and adult snails. All tests were conducted under semi-static conditions, with water renewal every other day in the 28-d tests or every 3.5 d in the 56-d, 35-d and 21-d tests. All tests were performed under a photoperiod of 14:10 hours light:dark. Snails were fed *ad libitum* with fresh organic lettuce (National AB–Agriculture Biologique and International Ecocert FR-BIO-01 certifications). The tests were conducted for different purposes (*e.g.*, range finding toxicity tests, evaluation of the suitability of the draft OECD protocols [6], and calibration of mechanistic effect models), at a different time, and therefore differed in their design as briefly explained in the following text (short descriptions of tests design is provided in Tables 5.1-3). Details regarding the frequency of observations are provided in Appendix 3 Table A1.

Two partial life-cycle tests with adult snails (with a duration of 28 d in the range finding test and 56 d in the toxicity test) were designed based upon the OECD recommendations [6], in order to check whether or not the proposed protocol allow demonstrating adverse effects of alkylphenols. In these two tests, we monitored both the effects of oestrogenic chemicals on exposed adults and on their non-exposed offspring. The 28-d range finding test was performed with five replicates of 25 snails; a water and a positive control (EE2) were included. In the 56-d toxicity test, we used six replicates of 30 snails; water, solvent, and positive controls were included.

The 35-d partial life-cycle test with sub-adults was specifically designed to best suit the needs for modelling within the Dynamic Energy Budget (DEB) framework [28], as demonstrated in Chapter 4. Similar to the tests with adults, here we also monitored performance of sub-adult snails and their non-exposed offspring. Note that in this test only four snails per treatment were used, including solvent and positive control (EE2), while 10 snails were used for water control. Detailed description of the test design is provided in Chapter 4.

Finally, a 21-d partial life-cycle test with embryos was performed. The purpose of this test was to assess the sensitivity of embryos toward EE2 (the effects of alkylphenols were not assessed). Egg clutches, laid within 24 hours by unexposed snails, were collected from the laboratory culture. Eggs were then extracted from their jelly-mass and exposed to EE2 in isolation. Tests were conducted with water and solvent controls. More details about the test design can be found in Chapter 3.

In the present paper all effects of chemicals are expressed as mean values unless otherwise stated. Note that in the 28-d and 56-d tests not all three alkylphenols were tested, and that in the 21-d test offspring was exposed only to EE2 (Table 5.1).

Table 5.2. Initial shell length of *L. stagnalis* and mean temperatures of the test medium in different tests.

Partial life-cycle tests			
28-d test with adults	56-d test with adults	35-d test with sub-adults	21-d test with exposed offspring
Shell length (mm ± standard deviation)			
25.6 ± 1.3	22.6 ± 1.1	19.6 ± 0.7	-
Temperature (°C ± standard deviation)			
22 ± 0.9	20.5 ± 0.6	22.2 ± 1.3	22 ± 1.3

Table 5.3. Summary of test endpoints monitored in partial life-cycle tests with offspring, sub-adults, and adults of *L. stagnalis*.

	Partial life-cycle tests			
	28-d test with adults	56-d test with adults	35-d test with sub-adults	21-d test with exposed offspring
	Treatments			
	4nNP Water and positive control (EE2)	NP Water, solvent, and positive control (EE2)	4nNP NP OP Water, solvent, and positive control (EE2)	EE2 Water control
Test endpoints for adults and sub-adults				
Survival	+	+	+	-
Shell length	+	+	+	-
Dry weight of soft body	-	+ [a]	-	-
Cumulated number of eggs per snail	+	-	+	-
Cumulated number of egg clutches per snail	+	+	+	-
Cumulated dry weight of egg clutches per snail	-	-	+	-
Number of eggs per clutch	+	-	+	-
Dry weight per egg clutch	-	+	+	-
Dry weight per egg	-	-	+	-
Size at first oviposition	-	-	+	-
Age at first oviposition	-	-	+	-
Test endpoints for non-exposed offspring				
Hatching success	+	-	-	-
Time to hatch [b]	-	+	-	-
Dry weight of hatchlings [b]	-	+	-	-
Shell length of hatchlings [b]	-	+	-	-
Frequency of abnormal eggs [c]	+	-	+	-
Frequency of polyembryonic eggs [c]	+	-	+	-
Frequency of unfertilized eggs [c]	+	-	+	-
Frequency of albumen-atrophied eggs [c]	+	-	+	-
Test endpoints for exposed offspring				
Hatching success	-	-	-	+
Time to hatch	-	-	-	+
Shell length of hatchlings	-	-	-	+

"+" = the endpoint was monitored; "-" = the endpoint was not monitored.
[a] The endpoint was monitored for NP, but not for the positive control; [b] egg clutches were collected from the 54th d of exposures; [c] the endpoint was assessed from egg clutches collected during the fourth week of the 28-d test or from the total number of collected clutches in the 35-d test.

5.2.3. Chemical analysis

Exposure medium was sampled at the beginning, mid-term, and end of the experiment. Three samples were taken per concentration, each sample (200 mL) consisting of the pooled water from two beakers. Samples were taken 15 minutes and 48 hours after water renewal and stored in glass flasks at -20°C until analysis. EE2 was extracted from water using C18 solid phase extraction (SPE) and quantified by high-resolution liquid chromatography-mass spectrometry (HPLC-MS/MS). Actual exposure concentrations of EE2 were calculated as the time-weighted average exposure concentrations (AEC) over the test period [29]. Obtaining the actual concentrations of alkylphenols in water is underway, and thus data on effects of alkylphenols and EE2 are presented and interpreted in terms of nominal concentrations.

To measure the internal concentration of EE2 in snails their body was removed from the shell. The fleshes were placed overnight at -80°C before lyophilisation and then 250 mg of dry weight was grinded. A liquid/solid extraction based on EPA method 1694 was used to extract compounds from flesh. Three successive extractions were performed. All fractions obtained were pulled in a final volume of 85 mL. Extracts were then concentrated using a rotary evaporation in a final volume of 30 mL to remove solvent. Before performing the SPE, samples were diluted in 200 mL of water to limit the impact of residual solvent with the cartridge. The SPE was performed using Strata® C18-E cartridge from Phenomenex (Torrance, CA, USA). The cartridges were eluted with 5 mL of methanol. The final eluates were evaporated under a gentle stream of nitrogen in a dry bath and reconstituted with methanol to a final volume of 20 µL. The analysis was then performed by HPLC/MS.

5.2.4. Statistical analysis

Effects of the chemicals were assessed using statistical tests as recommended for the analysis of ecotoxicity tests data [30]. To test for significant effects of the tested chemical, data were first checked for normality and homogeneity of variance, after which an appropriate statistical test was performed on various sample sizes (see Appendix 3 Tables A2-5). Data on performance of snails in water and solvent controls were assessed for differences. If no significant difference was found, the data were pooled and simply referred to as a control; otherwise, the solvent control was used for the comparisons with treatments. Analyses were performed using the SPSS (v.20) software (IBM SPSS, Armonk, NY), except for the Cochran–Armitage test where the JMP® (v.10) software (SAS Institute Inc., Cary, NC) was used. The significance level of all statistical tests was set to $\alpha = 0.05$. Highly significant probability of the test statistic, $0.001 < p < 0.01$ and $0.0001 < p < 0.001$, was highlighted on figures by two and three asterisks, respectively.

5.3. Results

5.3.1. Time-weighted average exposure concentrations of 17α-ethynyloestradiol and its body residue

Actual water concentrations of EE2 and its body residues (expressed as total EE2) were obtained from samples taken in the 56-d test. The results of the chemical analyses are summarized in Table 5.4, including the bioaccumulation factors (BAFs) of EE2. Mean time-weighted average exposure concentrations (AEC) corresponded to 35.9 ± 0.5% (SD) of the nominal concentrations. The BAFs varied between exposures, and their mean value was 64.3 ± 79 (SD).

Table 5.4. Nominal water concentrations, time-weighted average exposure concentrations (AECs), the body residue of EE2 and its bioaccumulation factors (BAFs) after a 56d exposure period.

Nominal concentrations in water (µg EE2/L)	**AECs of EE2** (µg EE2/L)	**Body residue** (µg EE2/kg wet weight)	**BAF**
0.01	N.D.	4.5	N.D.
0.055	0.02	4	200
0.3	0.107	2.95	27.6
1.7	0.608	4	6.58
9.1	3.29	177	53.8
50	17.6	533	30.3

N.D. = not determined

5.3.2. Effects of toxicants

In this chapter we show in figures only the results of experiments where a significant effect of the chemicals occurred. Tests results are summarised in Table 5.5, while the complete overview can be found in Appendix 4.

5.3.2.1. Effects of 17α-ethynyloestradiol

The cumulated number of clutches per snail in the 56-d toxicity test with adult snails was significantly lower in the solvent control relative to the water control (t-test). Therefore, in the 56-d test, the cumulated numbers of clutches per snail in EE2 treatments were compared with the solvent control. The results of this comparison show an adverse effect of EE2 on this endpoint at the highest concentration (Figure 5.1; Jonckheere-Terpstra test). No significant effect was recorded on any other endpoint monitored in all tests with EE2.

Table 5.5. No-observed effect concentration (NOEC) values (µg/L) obtained for different endpoints and life stages in different treatments of partial life-cycle tests with *L. stagnalis*.

	Partial life-cycle tests			
	28-d test with adults	56-d test with adults	35-d test with sub-adults	21-d test with exposed offspring
	Test chemicals and type of test controls applied			
	4nNP Water and positive control	NP Water, solvent, and positive control	4nNP NP OP Water, solvent, and positive control	EE2 Water control
	NOEC values (nominal concentrations in µg/L)			
Test endpoints for adults and sub-adults				
Survival	No effect	No effect	↓ 500 (NP)	-
Mean shell length	No effect	No effect	No effect	-
Mean dry weight of soft body	-	No effect [a]	No effect	-
Cumulated number of eggs per snail	No effect	-	↓ 200 (NP) ↓ 500 (OP)	-
Cumulated number of egg clutches per snail	No effect	↓ 91 (NP) ↓ 9.1 (EE2)	↓ 200 (NP) ↓ 500 (OP)	-
Cumulated dry weight of egg clutches per snail	-	-	↓ 200 (NP) ↓ 500 (OP)	-
Mean number of eggs per clutch	No effect	-	No effect	-
Mean dry weight per egg clutch	-	No effect	No effect	-
Mean dry weight per egg	-	-	No effect	-
Size at first oviposition	-	-	No effect	-
Age at first oviposition	-	-	No effect	-
Test endpoints for non-exposed offspring				
Hatching success	No effect	-	-	-
Mean time to hatch	-	No effect	-	-
Mean dry weight of hatchlings	-	No effect	-	-
Mean shell length of hatchlings	-	↑ 17 (NP) No effect (EE2)	-	-
Frequency of abnormal eggs	No effect	-	No effect	-
Frequency of polyembryonic eggs	No effect	-	No effect	-
Frequency of unfertilized eggs	No effect	-	No effect	-
Frequency of albumen-atrophied eggs	No effect	-	No effect	-
Test endpoints for exposed offspring				
Hatching success	-	-	-	No effect
Mean time to hatch	-	-	-	No effect
Mean shell length of hatchlings	-	-	-	No effect

"-" = the endpoint was not monitored; "↓" = the chemical has an inhibitory effect on the endpoint; "↑" = the chemical has a stimulatory effect on the endpoint.

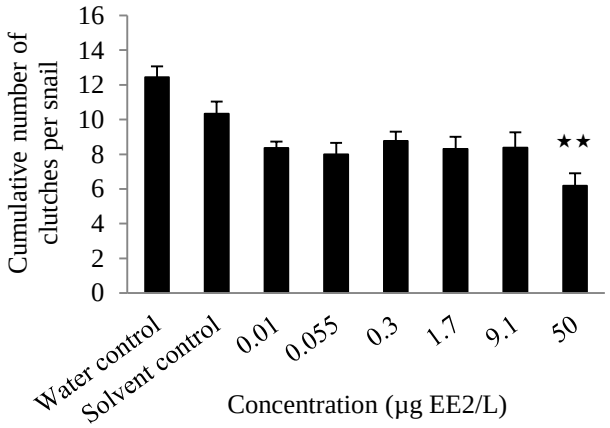

Figure 5.1. Effect of EE2 on the cumulative number of egg clutches per snail in the 56-d test with adult snails. Error bars represent the standard error. Asterisks indicate a significant difference ($0.001 < p < 0.01$) between data obtained in treatments and the control.

5.3.2.2. Effects of 4-nonylphenol and 4-n-nonylphenol

Survival of sub-adults decreased significantly (Cochran-Armitage test) by 50 ± 30% (SE) at 1000 µg NP/L in the 35-d test with sub-adult snails (Figure 5.2).

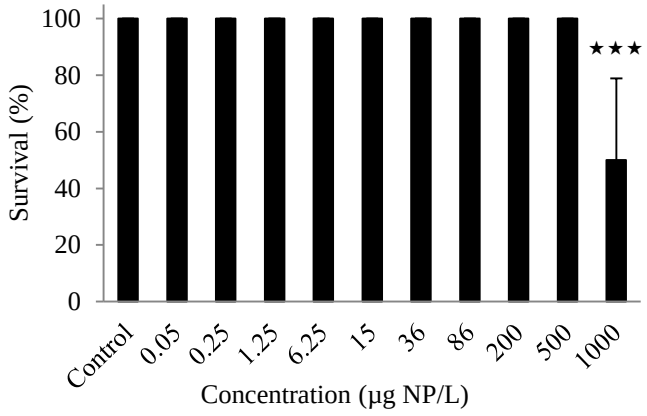

Figure 5.2. Effects of NP on the survival of sub-adults in the 35-d test. Error bar represents the standard error. Asterisk indicates significant differences ($0.0001 < p < 0.001$) between data obtained in treatments and in the control.

In the 35-d test, the cumulated number of egg clutches per snail decreased by 94 and 100% at 500 and 1000 µg NP/L (Mann-Whitney test with Bonferroni-Holm correction), respectively, and by 43% at 500 µg NP/L in the 56d test with adults (Jonckheere-Terpstra test; note that comparisons of the data in the 56-d test are made relative to the solvent control). Furthermore, in the 35-d the test a decrease of the cumulated dry weight of clutches by 95 and 100% at 500 and 1000 µg NP/L (Mann-Whitney test with Bonferroni-Holm correction), respectively, was also observed. At the same concentrations in the 35-d test, EE2 affected the cumulated number of eggs, decreasing the reproductive output by 93 and 100% (Mann-Whitney test with Bonferroni-Holm correction). Note a large fluctuation in the data on reproduction over treatments in the 35-d test, although not significant.

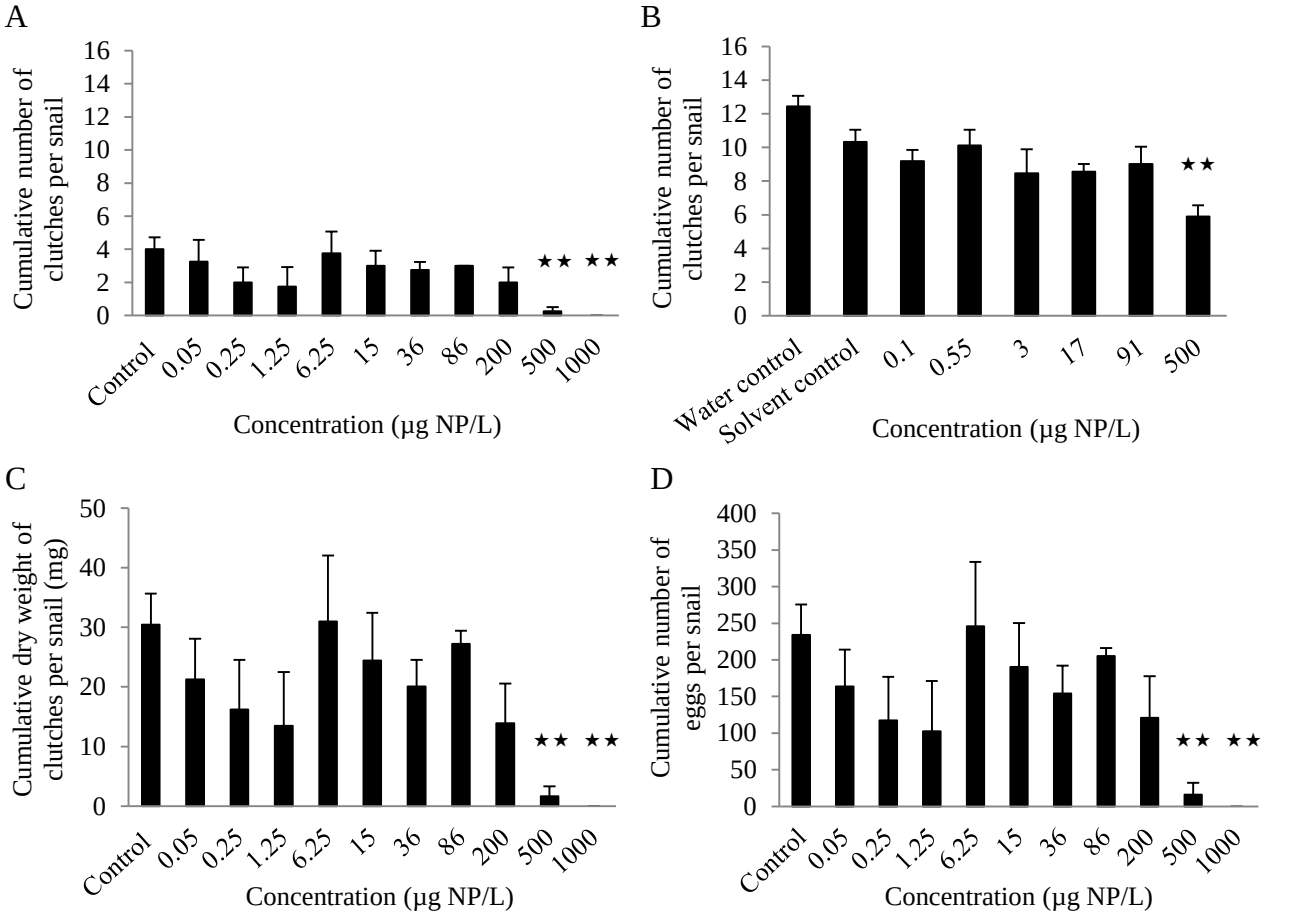

Figure 5.3. Effects of NP on the cumulated number of clutches per snail in A) the 35-d and B) the 56-d tests, C) on the cumulated dry weigh of clutches per snail in the 35-d test, and D) on the cumulated number of eggs per snail in the 35-d test. Error bars represent the standard error. Asterisks indicate significant differences ($0.001 < p < 0.01$) between data obtained in treatments and the control (35-d test) or the solvent control (56-d test).

Snails exposed to 500 µg NP/L during 54 d produced offspring that had 10% longer shells in comparison with the data from the control group (ANOVA with Dunnett's post hoc test; Figure 5.4A). Offspring in the same treatment also had an increased dry weight (as a whole body with the shell) for 21% comparing with the control data (Figure 5.4B; the test design was not appropriate for obtaining data relevant for statistical analysis, as shown in Appendix 3 Table A4).

4nNP did not cause observable effects in any of the tests with offspring, sub-adult and adult snails.

5.3.2.3. Effects of 4-tert-octylphenol

Reproduction endpoints were significantly modified only at the highest exposure concentration of OP, *i.e.* at 1000 µg/L. OP caused a 90% decrease of the cumulated number of eggs, a 88% decrease of the cumulated number of clutches, and a 90% decrease of the cumulated dry weight of clutches (Figure 5.5A-C; the significance of the reported results was confirmed with the Mann-Whitney test with Bonferroni-Holm correction). There is a large variation in reproduction data in OP treatments.

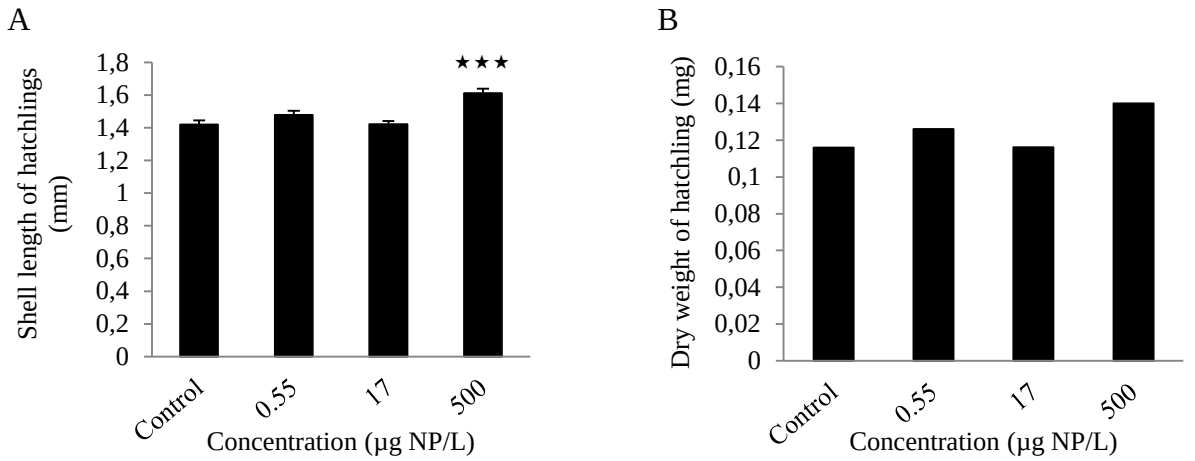

Figure 5.4. Effects of NP on A) the shell length and B) the dry weight of hatchlings produced by snails exposed to the toxicant during 54 d. Error bars represent the standard error. Asterisks indicate a significant difference ($0.0001 < p < 0.001$) between data obtained in treatments and the control.

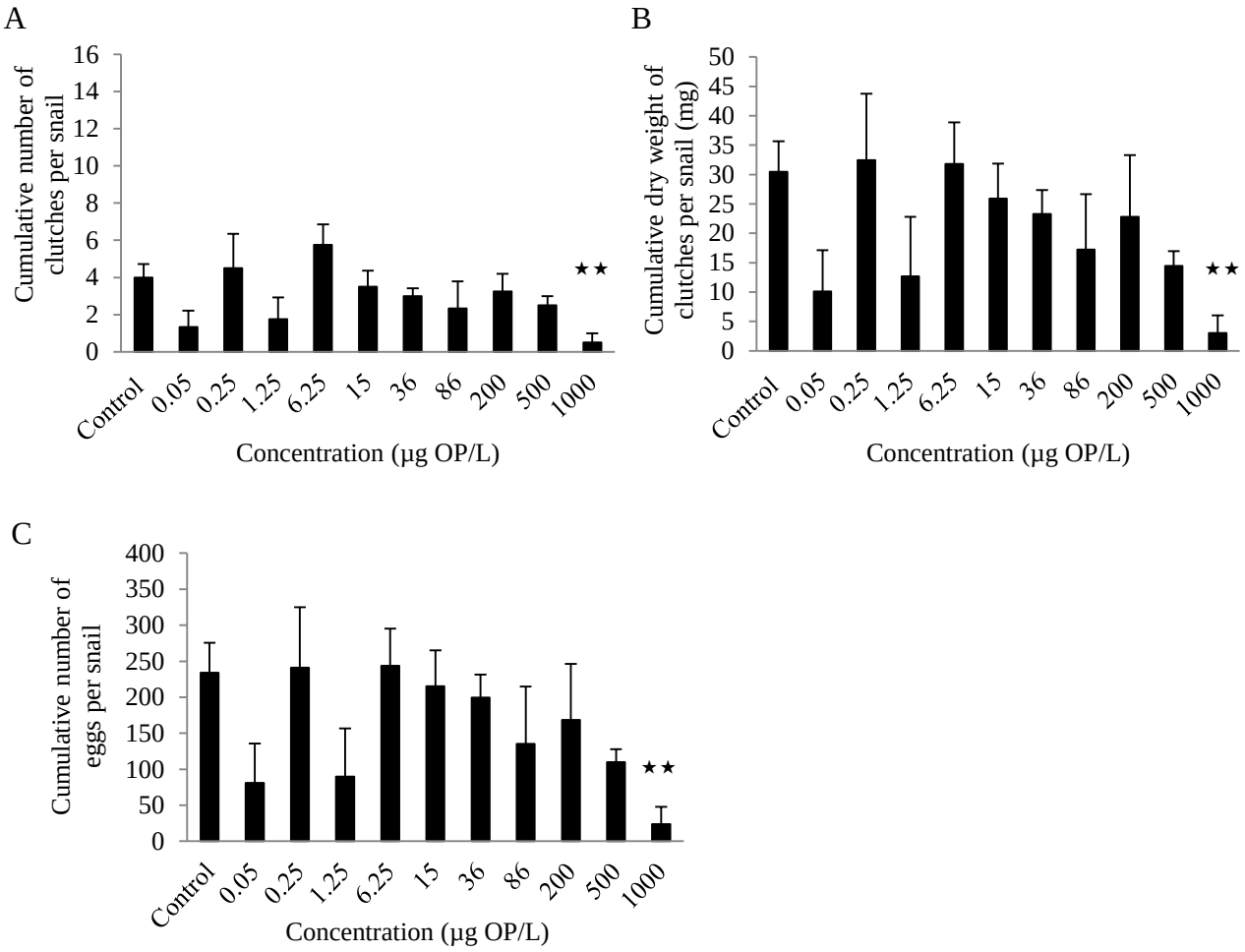

Figure 5.5. Effects of OP in the 35-d test on A) the cumulated number of clutches per snail, B) the cumulated dry weight of clutches per snail, C) the cumulated number of eggs per snail. Error bars represent the standard error. Asterisks indicate a significant difference ($0.001 < p < 0.01$) between data obtained in treatments and the control.

5.4. Discussion

AEC values suggest a relatively rapid loss of EE2 in water (Table 5.4). Chemical losses were due to different processes, including bioaccumulation by snails, as indicated by the mean value of the BAF of 64.3 for a 56-d exposure period. Complementary studies are required to see whether and how much EE2 was biotransformed in snail tissues, and to fully assess the toxicokinetics.

The effects of EE2 and the alkylphenols were generally consistent between the different tests we performed (which had different designs in terms of duration and number of tested snails). This demonstrates the robustness of reproduction toxicity testing with *L. stagnalis*. The amount of EE2 accumulated in snails had no effect on the individual performance, since none of the tested endpoints was affected at environmentally relevant concentrations in any of the partial life-cycle tests. NP affected survival of sub-adults only at a concentration as high as 1000 µg/L (Figure 5.2), while in the 56-d and the 35-d tests, 500 µg NP/L induced significant effects on some of the reproduction endpoints (Figure 5.3). In the 35-d test, OP exerted negative effects on the cumulated number of eggs, egg clutches, and dry weight of eggs per snail only at 1000 µg/L. A large variation of data on reproduction makes it difficult to establish a concentration-response for NP and OP in the 35-d tests. 4-n-nonylphenol did not induce any effect on any of the tests endpoints. Overall, our results suggest that exposure to the selected chemicals did not have significant consequences on the selected life-cycle traits at environmentally relevant concentrations. Significant effects of EE2, NP and OP were observed only at very high concentrations, suggesting overt toxicity rather than endocrine disruption.

The lack of adverse effects with environmentally relevant concentrations of oestrogenic chemicals in our study is in accordance with the observations of Giusti and co-workers [31] for EE2 and chlordecone, and with those of Czech and co-workers [15] for NP. However, our study does not confirm the findings of Segner and co-workers [32] who observed a negative impact of EE2 on *L. stagnalis*. These authors reported a variety of effects of EE2 on metabolism, histopathology, development, growth, and reproduction of the snail at concentrations ranging from 0.05 to 1 µg EE2/L. The relevance of these findings is difficult to assess because the limited description of the test design provided by the authors does not allow for a detailed interpretation of their results. Regarding the effects of NP, 5- and 8-week exposure to concentrations up to 200 µg NP/L did not affect any of the test endpoints in our study, which is in a good agreement with the observations made by Czech and co-workers [15], who exposed snails to 100 µg NP/L during seven weeks.

Other hermaphroditic pulmonates, such as *Radix balthica* and *Physa pomilia*, do not seem to be adversely affected by environmentally relevant concentrations of *e.g.* EE2. Partial and full life-cycle laboratory tests with these species have demonstrated no effect of this chemical on reproduction endpoints [10-12]. Yet, low concentrations of EE2 and other oestrogenic chemicals have raised concern due to their ability to induce stimulatory effects on the reproduction of prosobranchs, *e.g. P. antipodarum, Marisa cornuarietis, and Nucella lapillus* [7-9]. Therefore, we wonder why oestrogenic chemicals might induce effects on the reproduction of prosobranch, but not of pulmonate gastropods.

Our first hypothesis for the absence of effects on life-cycle traits in exposed gastropods is linked to the toxicokinetics of the chemicals. For example, many aquatic snail species can maintain a constant level of free testosterone by conjugating its excess to a fatty acid ester. The lack of effect of waterborne testosterone on the snails *Ilyanassa obsoleta* and *L. stagnalis* was attributed to this homeostatic mechanism, as maintenance of constant levels of free testosterone and increased levels of esterified testosterone were observed in exposed snails [33, 34]. On the opposite, exposure of females of *M. cornuarietis* to waterborne testosterone does not significantly modulate the level of esterified testosterone in the body [35]. The lack of homeostatic regulation of testosterone levels in this prosobranch might be the reason for the appearance of imposex in females exposed to this steroid. Moreover, EE2 in the same species elicits the expected oestrogenic effect, *i.e.* an increase of fecundity [36], which could also be explained by the lack of homeostatic regulation of steroids

concentrations. Indeed, the same enzyme, acyl-coenzyme A acyltransferase, is responsible for the biotransformation of both testosterone and 17β-oestradiol into fatty acid esters in snails [35, 37]. Absence of this enzyme in some prosobranch species might thus prevent them from detoxifying steroids or chemicals that mimic steroids. The presence of this enzyme in *L. stagnalis*, could explain that this species (and putatively other gastropods) can esterify excess of testosterone and possibly EE2. However, this mechanism of homeostatic regulation of EE2 in *L. stagnalis* is speculative. Similar studies as conducted for testosterone by Giusti and co-workers [33] are required in order to validate this hypothesis.

The toxicokinetics of alkylphenols differ from that of EE2 in *L. stagnalis*. Indeed, they are not esterified but biotransformed and excreted from the organism [38-40]. Biotransformation kinetics varies among alkylphenols due to differences in the branching degree of the alkyl group. Linear chains of alkylphenols are more readily biodegraded relative to branched ones [16]. Therefore, they are expected to be eliminated faster from the body, and thus to induce less toxicity. Our results support this hypothesis because 4nNP (with the linear alkyl chain) did not affect any of the tested endpoints, while NP and OP (both with branched alkyl chains) affected survival and some reproduction endpoints at very high concentrations (Table 5.5 and Figures 5.2, 5.3, and 5.5).

Apart from esterification and biotransformation, EE2 and alkylphenols can be removed from the adult body by transferring the chemical to the eggs: this is a common process in aquatic organisms (*e.g.*, [41]). In our experiments, NP showed a potential to affect embryogenesis when adults were exposed to the chemical during 54 d. Hatchlings originating from snails exposed to 500 µg NP/L had larger shell lengths and increased body dry weight relative to the control snails. Observing effects on embryos from exposed parents in our study points at alternative hypotheses: (i) embryos may be recipients of NP from their parents and/or (ii) embryos might not be directly affected by NP, but parents stressed by the toxicant may allocate more energy in their offspring, thus producing fewer but bigger embryos.

Literature studies suggest that a prolonged exposure to alkylphenols might cause negative effects on snails. Indeed, exposure to 100 µg NP/L during 12 weeks affected reproduction and caused histopathological damage in *L. stagnalis* [15]. Our studies involved a maximum exposure period of eight weeks. The results suggest that alkylphenols and EE2 in *L. stagnalis* might be regulated through biotransformation and esterification of the chemicals. However, our studies did not allow elucidating whether or not esterification of EE2 or elimination of alkylphenols are complete, or if the body burden increases slowly with exposure duration. In the latter case, a longer test duration might be required in order to demonstrate adverse effects of these chemicals.

The second hypothesis for observing no adverse effects of the oestrogenic chemicals in our toxicity tests may lie in the toxicodynamic processes, *i.e.* in the interaction between the studied toxicants and their putative target site (the ER or another hormonal receptor, under the endocrine disruption assumption). To date, the ERs have been identified in several prosobranch species such as *P. antipodarum* [17], *M. cornuarietis* [18], and *N. lapillus* [19], or pulmonates like *L. stagnalis* (M.A. Coutellec and co-workers, unpublished data), *L. ollula* [42], and *R. balthica* [11]. In *M. cornuarietis*, both EE2 and bisphenol A (another alkylphenol) stimulated fecundity (EE2 exhibiting the strongest effect, which supports the hypothesis of a higher potency of EE2 relative to alkylphenols) [36]. Furthermore, exposure of *e.g. P. antipodarum* and the mussel *Mytilus edulis* to 17β-oestradiol or EE2 induced an increase of the snail fecundity as well as an increase in the expression of the ER mRNA transcripts in both species [43, 44]. Based upon these results, authors have hypothesised that 17β-oestradiol or EE2 might be responsible for the observed increase in fecundity by binding to the ER. Some attempts were made to demonstrate that exogenous 17β-oestradiol can bind to the ER in these species. Yet, no evidence for such binding could be provided. The actual ligand of the ER in molluscs is unknown, as well as the mechanism by which the expression of the ER mRNA is increased by 17β-oestradiol or EE2. Some authors suggest that the activity of the ER might be constitutive, *i.e.* binding of the oestrogen ligand to the receptor is not needed for its activation (*e.g.*, [17] and references therein). It might also be that the target of

oestrogenic chemicals is not the ER itself but other biomolecules that interfere with the expression mechanisms of the mRNA that code for the ER. Finally, the study of Stange and co-workers [43] highlighted the existence of a wide range of possible targets in the oestrogen genomic and non-genomic signalling pathways. Even a slight difference in these pathways between pulmonate and prosobranch snails may lead to observed difference in the effect pattern of oestrogenic chemicals.

Our last hypothesis is that the ER does not have, or has a limited/different role in the development, growth, and reproduction of *L. stagnalis*, as compared to other molluscs. Some steroids are involved in digestion processes, others are used as communication substances or have a role in the regulation of sterol homeostasis or embryonic development [45]. Therefore, the ER of *L. stagnalis* may actually serve some of the related functions. It also cannot be excluded that other receptors than the ER bind steroids [46]. In that case, it would be plausible that prosobranchs have receptors that bind oestrogenic chemicals and express effects on reproduction while pulmonates do not have them or have different ones, and so forth (see [46]).

In view of the discussion above, it is currently not possible to establish the mechanism by which the oestrogenic chemicals, namely EE2 and alkylphenols, affect *L. stagnalis* and elicit their effects at high concentrations. Current knowledge about the functioning of the endocrine system of *L. stagnalis* is limited, which prevents us from gaining better understanding of the effects of endocrine disruptors in this species. Hopefully, the project PLAYLIST, which is underway in our research group, will contribute to filling the knowledge gap on EDs binding receptors in *L. stagnalis* and ensure better understanding of endocrine disruption in molluscs.

For the testing of oestrogenic chemicals, the use of 17β-oestradiol or EE2 as a positive control was proposed in several literature studies, under the assumption that these chemicals will stimulate reproduction due to their binding to the molluscan ER. Our study has shown that neither EE2 nor alkylphenols induced any increase in the fecundity of *L. stagnalis*, which support findings from other studies on the effects of EE2 in pulmonates [10-12]. Based upon these results, the validity of using 17β-oestradiol or EE2 as positive controls in reproduction toxicity tests with *L. stagnalis* is disputable.

Oestrogenic chemicals are expected to elicit stimulatory effects on *e.g.* fecundity. It has to be highlighted that such stimulatory effects on reproduction are always associated with increased feeding rate or a decreased investment in another life-cycle trait (*e.g.*, growth) [47]. Therefore, it is advisable to additionally monitor the corresponding test endpoints in future studies on oestrogenic effects. This may not only provide complementary information about the effects, but it would also be relevant for the ERA of chemicals for aquatic invertebrates [48], as effects on *e.g.* growth (associated with those on reproduction) can determine the level of the chemical impact on sustainability of populations of aquatic invertebrate [49]. Furthermore, classical summary statistics in ecotoxicology such as NOEC or EC_x does not allow biological interpretation of chemical effects on organisms [50]. Understandings the effects of chemicals, including those of EDs, can be facilitated by using toxicokinetic-toxicodynamic modelling approaches. This would, indeed, require a different test design than the one recommended for the OECD standardisation of current test protocols for molluscs [6].

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6. General discussion

In this thesis, I investigated the effects of putative endocrine disruptors on the life-cycle traits of the great pond snail *L. stagnalis*. I also explored experimental design to accommodate TKTD models. In Chapter 2, effects of tributyltin (TBT) and triphenyltin (TPT) were evaluated. TBT elicited effects on snails that could potentially be linked to endocrine disruption. Findings from Chapter 3 about the most appropriate experimental design for TKTD modelling helped to further design toxicity tests to study mechanisms of effects of putative EDs. By using the DEBkiss model, I argue that TBT affects the feeding rate of snails (Chapter 4), which decreases growth and consequently affects reproduction. In Chapter 5, I evaluated the effects of oestrogenic chemicals (as defined in vertebrates) and found that the studied chemicals did not impact snails significantly at environmentally relevant concentrations. In the following, I will discuss possible reasons for not observing the effects of these vertebrate-like steroids in snails. This forms the basis for further discussion about mechanisms of observed effects of TBT and TPT in snails. I also evaluate the usefulness of the DEBkiss model for the analysis of the effects of putative EDs in the great pond snail. Finally, recommendations for improvement of test designs to obtain optimal data sets for different types of ecotoxicological analyses are provided.

6.1. Steroid signalling pathways and endocrine-mediated effects of toxicants in *L. stagnalis*

I investigated whether synthetic steroid hormones such as 17α-methyltestosterone (MT, Appendix 5) and 17α-ethynyloestradiol (EE2) might exert androgenic or oestrogenic effects in *L. stagnalis*, respectively, similar to those observed in vertebrates and some prosobranch snails. Observing biological effects of these two chemicals in *L. stagnalis* might support the hypothesis that steroid pathways are somehow involved in the hormonal control of the development, growth, and reproduction. Furthermore, if exposure of the great pond snail to organotins (androgenic chemicals) or alkylphenols (oestrogenic chemicals) results in the effects similar to those elicited by steroids, it is an indication of endocrine disruption.

We exposed snails to MT (Appendix 5) or EE2 (Chapter 5) to test the hypothesis of the involvement of vertebrate-like steroid pathways in the regulation of the development, growth, and reproduction of *L. stagnalis*. At environmentally relevant concentrations, no adverse effects of these chemicals were observed. Results from the toxicity tests with MT confirmed previous results of Giusti and co-workers who observed no effects of testosterone on cumulated number of eggs and clutches after 21-d exposure [1]. In EE2 treatments, only a slight decrease of the cumulative number of clutches per snails was recorded at 50 µg EE2/L after 56-d exposure. This is approximately 100 to 1000 fold higher than effective concentrations observed in some prosobranchs [2], which suggests overt toxicity rather than endocrine mediated effects of EE2 in *L. stagnalis*. The same was concluded in a similar study on effects of EE2 on *L. stagnalis* [3]. In addition, our tests with sub-adult snails and embryos did not reveal any negative impact of EE2 even at the highest tested concentration of EE2 of 80 µg/L.

The explanation for the lack of effects of EE2 and MT might lay in their toxicokinetics or toxicodynamics. Giusti and co-workers [1] also did not observe effects of testosterone on the reproduction of *L. stagnalis*, which was explained by snail's ablity to conjugate the excess of testosterone with fatty acid esters so as to keep free testosterone levels constant. This allows snails to maintain physiological homeostasis because the esterified testosterone is biologically inactive. The same explanation may hold for MT because MT is a synthetic form of testosterone, which is the natural ligand of the androgen receptor (AR) in vertebrates. However, since the AR has never

been identified in molluscs [4] it is very unlikely that the AR constitutes a pathway through which MT could have exerted its effects.

Some mollusc species are able to esterify 17β-oestradiol [5], so the same mechanism as for testosterone may explain our observations of EE2 effects. Indeed, esterification of both 17β-oestradiol and testosterone is under the control of the same enzyme in some molluscs, *e.g.* in the prosobranch *Ilyanassa obsoleta* and the oyster *Crassostrea virginica* [6, 7]. Yet, we do not have empirical evidence for the ability of *L. stagnalis* to esterify EE2. Our preliminary analysis did not confirm presence of the esterified EE2 form in snail tissue and haemolymph following a 56-d exposure period to this chemical – only free EE2 was found (A. Barsi, unpublished data). Contrary to the AR, in some *Lymnaeidae* the presence of the molluscan oestrogen receptor (ER) has been confirmed [8, 9]. There are also encouraging results from our research group that the ER is present in *L. stagnalis* (M.A. Coutellec and co-workers, unpublished data). It could be expected that the molluscan ER binds 17β-oestradiol, which is the natural ligand of the vertebrate ER. However, such a ligand-binding possibility for EE2 (and other putative oestrogenic chemicals) remains to be demonstrated in *L. stagnalis*. It is possible that ligands for the ER in molluscs are not steroid-like ligands. Some authors have suggested that the activity of this ER receptor could be constitutive [10]. Moreover, it could also be that the ER in *L. stagnalis* has a completely different role than the ER of vertebrates, and that its potential activation does not have consequences on the development, growth and reproduction. Indeed, steroids in molluscs may serve other functions than hormonal, *e.g.* some steroids are involved in digestion processes, others are used as communication substances or have a role in the regulation of sterol homeostasis [11].

Overall, our results suggest that the vertebrate-like steroids are probably not involved in the pathways by which oestrogenic and androgenic chemicals might elicit their effects in the great pond snail. Therefore, EE2 or MT should not be used as positive controls in toxicity tests with *L. stagnalis*, since they probably do not interfere with the endocrine system in this species. In vertebrate studies on endocrine disruption, vertebrate hormones are used as positive controls. Likewise, exposing snails to their own hormones as listed in *e.g.* [12], could be a good starting point for identification of chemicals that might be used as positive controls in toxicity tests for studying endocrine disruption in *L. stagnalis*.

In the following text, I explore other possible mechanisms by which effects of the putative endocrine disruptors TPT and TBT might be explained. I focus on effects of these organotins because they have been considered as androgenic chemicals in prosobranch snails and did induce effects on *L. stagnalis* in my experiments.

6.2. Mechanisms of the effects of organotins in *L. stagnalis*

It has been demonstrated that the great pond snail is sensitive to low concentrations of TBT (Chapters 2 and 4) and TPT (Chapter 2). The sensitivity of reproduction endpoints of *L. stagnalis* in toxicity tests I conducted was similar to that reported in the literature for this and other species [13, 14]. Furthermore, TBT and TPT did not induce significant non-monotonic concentration-responses. Yet, the effect pattern of these chemicals was different. Here, I first provide a brief overview of the main results (including some evidence on TPT effects that has not been presented in previous chapters (Appendix 6)), and subsequently discuss the findings.

TPT negatively affected survival of adults at 2.63 µg Sn/L, and their oviposition and fecundity at 1 µg Sn/L. Growth, expressed as shell length, was not affected and the snails did not produce significantly more abnormal eggs (Chapter 2). I also exposed sub-adult snails to TPT in a separate experiment (Appendix 6). TPT had an impact on the survival of sub-adults at 1.25 µg Sn/L, and on their growth and reproduction at 0.91 µg Sn/L. This lowest observed effect concentration was only 1.4 times lower than the lethal concentration. TBT did not affect survival of adult snails. Growth and reproduction were more sensitive endpoints than survival, *i.e.* the lowest-observed effect

concentrations (LOECs) for shell length were 0.48 and 0.53 µg Sn/L, the LOECs for the oviposition were 0.22 and 0.53 µg Sn/L, and the LOECs for fecundity were 0.22 and 0.53 µg Sn/L (Chapters 2 and 4, respectively). Particularly sensitive endpoint to TBT was the frequency of polyembryonic eggs laid by exposed snails, which was significantly increased at 0.05 µg Sn/L. In sub-adults, TBT increased mortality at 1.25 µg Sn/L, though the intensity of this effect seems to be smaller than for TPT (*e.g.* it took more days for TBT to reach similar effects as of TPT, and TBT concentration of 1.25 µg Sn/L did not kill all the snails while TPT did). Negative effects on growth and reproduction occurred at 0.26 µg Sn/L, which is almost 5 times lower than the lethal concentration.

Further, I investigated whether or not the pattern of TPT and TBT effects, as observed in sub-adult and adult snails, would be similar to the pattern observed in embryos. Therefore, I conducted an experiment in which isolated eggs were exposed to TPT during 21 d (Appendix 6). The hatching success decreased already at the lowest tested concentration (0.01 µg Sn/L), and the decrease was significant at 0.26 µg Sn/L. The hatchling size (shell length) significantly decreased at 0.26 µg Sn/L, while the time to hatch significantly increased at TPT concentration as low as 0.05 µg Sn/L. This is the concentration at which polyembryony was increased when adult snails were exposed to TBT (Chapter 2). In the 21-d TBT toxicity test with embryos (Chapter 4), hatching success was affected at 0.91 µg Sn/L, which is similar to the LOEC for survival of sub-adults of 1.25 µg Sn/L (Chapter 4). These results indicate that embryos are much more sensitive to TPT than to TBT. TPT concentration of 0.05 µg Sn/L was sufficient to increase the time to hatch, while the LOEC of TBT for all endpoints in the embryo test was approximately 18 times higher. Therefore, the effects of the two chemicals on embryos differ, but their order of toxicity is reversed in comparison to the effects in sub-adults and adults.

Results presented in Chapter 2 together with the additional data (Appendix 6) suggest that TPT elicits overt toxicity on adults and sub-adults of *L. stagnalis.* This can be concluded because TPT concentrations that affect growth and/or reproduction are only slightly lower than the lethal concentrations. Conversely, sub-lethal endpoints in TBT tests with adults and sub-adults were much more sensitive than survival, which may be an indication for endocrine disruption of TBT in *L. stagnalis* (Chapters 2 and 4). Increased frequency of polyembryonic eggs also supports the hypothesis of endocrine disruption of TBT in adult snails, although more testing is needed to confirm the consistency of TBT effects on this endpoint (egg abnormalities were not detected when the tests started with sub-adults, and were not equally detectable in all the tests we conducted in adults). It was suggested in Chapter 2 that the difference in the alkylation of the two organotins might be a reason for the observed deviations in response patterns of snails. This might also explain the effect patterns observed in embryos. However, it is not clear how such differences in the molecular structure of organotins might lead to the observed different effects within the various life-stages (*i.e.*, adults and sub-adults versus embryos) of *L. stagnalis.*

Generally, there are two conflicting hypotheses about mechanism of action of organotins, both aiming to explain imposex development in prosobranch snails. The first hypothesis states that elevated levels of testosterone are the main cause of imposex. However, free testosterone levels did not increase when adults of *L. stagnalis* were exposed to TBT [1]. Moreover, as mentioned earlier, the AR has not been identified in molluscs [4], so that it is unlikely that TBT interacts with the AR in snails. The second hypothesis explains imposex in prosobranchs by the involvement of the retinoid X receptor (RXR) signalling pathways, which has recently received more attention by scientists [15]. In many prosobranchs it has been shown that imposex development was followed by a significant increase of the expression of the RXR. The natural ligand of the RXR is retinoic acid (RA). Based upon results from *in vitro* studies on the rock shell *Thais clavigera* TPT and TBT have a very similar affinity to the RXR as RA has [15]. Furthermore, all three chemicals similarly stimulate the transcription activity of the RXR and induce imposex development [16]. Nevertheless, in some snails, such as the dog whelk *Nucella lapillus* and *T. clavigera*, two isoforms of the RXR are present, one being more intensely activated by RA than the other [16, 17]. Unfortunately, organotins have been tested only on the the isoform that showed higher activity in the presence of

RA. The RXR has been identified in embryos and other life stages of *L. stagnalis* [18] and we have shown that TBT induces adverse effects on all life stages. It can be hypothesised that in *L. stagnalis* two (or more) RXR isoforms are present, of which transcriptional activity might be differently stimulated by TPT and TBT. Moreover, these hypothetic isoforms might (or might not) be present in the different life stages. Altogether, this could explain the difference in responses of snails and embryos.

In this thesis, I focused on TBT effects in *L. stagnalis*, as much more data on this chemical were available from my experiments and the literature. Experimental observations and the DEBkiss analysis (Chapters 2 and 4) suggested that TBT reduces feeding in adults and sub-adults. When less energy is available than optimally needed, this leads to a decrease of the growth rate and a smaller ultimate size. Therefore, TBT does not seem to affect reproduction directly: the decreased reproductive output is a consequence of decreased food intake and a smaller body size. As the effects of TBT in adults and sub-adults where more pronounced than those in embryos, we hypothesised that a specific aspect of feeding on lettuce was affected. Assuming that the RXR is involved in mediation of the effects, the possible existence of two isoforms of the RXR would suggest different intrinsic effects of TBT on (i) embryos and (ii) sub-adults and adults.

More research is needed in order to elucidate the mechanisms of toxic effects of organotins in *L. stagnalis*. The RXR is a good candidate to be further studied. Experiments conducted at the sub-individual level and that involve "omics" techniques in combination with apical tests and TKTD modelling would shed light on the mechanisms of toxicity of organotins and other putative EDs.

6.3. Evaluation of the DEBkiss model for data analysis

6.3.1. Advantages of the DEBkiss model over descriptive analyses

DEB theory sets the rules for the acquisition of energy from the environment and its subsequent allocation to metabolic processes that define development, growth, and reproduction of an individual organism over its entire life cycle. Toxic effects on growth and reproduction must have underlying changes in these allocation rules. DEB models are well suited for the analysis of toxicity test data over time (at least for those endpoints that have a clear energetic cost associated with it). The no-effect concentration (NEC) is perhaps the most ecotoxicologically relevant DEB parameter (and certainly the easiest to interpret). It represents the external concentration that does not lead to an effect on a metabolic process, even after prolonged exposure. This parameter has been recognised as a valid summary statistic in the context of environmental risk assessment (ERA) [19, 20]. However, DEB models have been used more in ecotoxicological research than in risk assessments. This is partly because risk assessors need guidance on how and when to apply models (or when to accept model results) [21], and partly due to the (perceived) complexity of DEB models. The simplified DEBkiss model that I applied in my thesis has recently been developed with, as one of the aims, the facilitation of the application of DEB theory in ecotoxicology and in the ERA of chemicals [22]. Compared to the standard DEB animal model [23], DEBkiss is based on several simplifying assumptions regarding the animals' metabolism and physiology (Appendix 1). These simplifications make the model most suitable for the analysis of data on invertebrates. Initially, the DEBkiss model was used to explain life-history traits of *L. stagnalis*, and effects of stressors such as food limitation and starvation [22]. Chapter 3 [24] is the first published example where this model has been used to study effects of chemicals. The model has also been applied to study the combined effects of temperature and cadmium on the springtail *Folsomia candida* [25]. More recently, the DEBkiss model has been used to explain the effects of a binary mixture on the nematode *Caenorhabditis elegans* [26].

The main advantage of a DEBkiss analysis over descriptive statistics is that the model takes into account all relevant information available from toxicity tests over time (the data for all endpoints and all time points are simultaneously used to estimate model parameters). By doing so, the model

provides ecotoxicologically relevant summary statistics, *i.e.* NECs for effects on survival and/or sub-lethal endpoints, which are, in contrast to the classical statistics like EC_x and NOEC, independent of exposure duration (see [27]). Indeed, it has been shown that the NEC provides a more precise estimation of toxicity than the standard statistics [28]. In Chapter 3, I show that NECs derived from DEBkiss are robust estimators for no-effect of toxicants (in this case acetone) on both lethal and sub-lethal endpoints. Furthermore, the DEBkiss model allowed combining data sets from various toxicity tests that consisted of the same, and also of different, types of test endpoints (Chapters 3 and 4). Moreover, the DEBkiss model was flexible enough to account for differences in test conditions, such as different temperature or solvent concentration. This allowed not only the estimation of NECs from various data sets, but also to identify the metabolic mode of action (mMoA) of toxicants.

Apart from calculating a simple summary statistic (the NEC), the DEBkiss parameters can also be used to make predictions for effects in untested scenarios (*e.g.*, under food limitation, different temperatures, pulsed exposure). The extrapolation power is one of the most valuable features of TKTD modelling [29]. When these models are based on mechanistic principles, they can be used to make educated guesses for the animal's response in realistic field conditions. This power can be exploited even further by linking DEB-based models to population ones (see [30, 31]).

To deduce the mMoA of a toxicant, it is usually sufficient that the toxicity test follows survival, growth, and reproduction over time. However, it is sometimes difficult to confidently identify the mMoA from such data sets [32]. For example, in Chapter 3, the mMoA of acetone could not be uniquely defined from data on adult and sub-adult snails only; it could be narrowed down to three different mMoAs which fitted the data equally well. Only when the exposure data on embryo development was included in the analysis, one mMoA could clearly be selected. Notably, the effects of acetone on embryos were described well with the same model and mMoA, and almost the same parameter set, as for sub-adults and adults. Predicting embryo development under control conditions needed tuning of some parameters (*e.g.*, the specific assimilation rate of the embryo and a lag-phase of 2.5 d for embryo development was assumed), which highlights the need for more detailed mechanistic study for this life stage. Furthermore, because the DEBkiss model has an explicit mass balance, it could deal with shrinking of snails, which was observed in several treatments (Chapter 3 and 4). However, shrinking of the animals is difficult to quantify as we only have a single estimate of dry weight for each individual snail (Chapters 3 and 4).

Regarding validation of the DEBkiss model, I demonstrate that the model is able to explain the observed effect patterns with reasonable accuracy, and that the precision of the predictions depends on the quality of the calibration data (Chapter 3). The model predictions were sensitive to small deviations in the values of toxicological parameters across different calibration data sets. Indeed, more thorough "evaludation" of the DEBkiss model is needed to fully assess its reliability and potential for the use in the ERA of chemicals [33].

6.3.2. Application of DEBkiss for the analysis of endocrine disruption

Analysing the effects of TBT in DEBkiss did not require adding any new assumption about the mMoA of the putative ED (Chapter 4). TBT effects were described with a regular mMoA; a decrease of the feeding rate. Minor model adaptations were added to account for temperature differences, saturating effects of TBT, and for apparent differences in bioavailability between the tests (presumably due to different solvent concentrations).

In Chapter 3, a stimulatory effect of acetone on growth (sub-adults) and reproduction (sub-adults and adults) over the tests duration was observed. As the effect did not occur in the same treatments for both size groups, we concluded that it was probably an experimental artefact. However, in Chapter 4 in the test with sub-adult snails, the lowest TBT concentration (0.01 µg Sn/L) showed a stimulatory effect on reproduction, which was accompanied by an inhibitory effect on growth in the same treatment. This could be considered as a non-monotonic dose response, although it requires

confirmation in a test with a higher resolution of tested concentrations (*e.g.*, in the range from 0 to the first LOEC). Furthermore, since only a small number of animals per treatment was used (four snails), the reason for this observation could easily be the animals' variability or an experimental artefact.

If this non-monotonic dose response pattern is indeed due to TBT toxicity, it could be captured by the DEBkiss model through a change in the value of the parameter κ (*i.e.*, the fraction of assimilates allocated to soma, see [34]). A decrease of κ results in an increased cumulative number of eggs, but also in a decrease of the growth (Figure 6.1). It also cause a smaller size at maturation when comparing to non-treated animals (see [34] and Appendix 1 for calculations). To investigate the plausibility of this additional mMoA at low exposure, I performed a simulation in which a value of the parameter κ differed between control and treated snails in the lowest exposure concentration. It can be seen in Figure 6.1 that the model fits the patterns in the data well (parameter values are presented in Table 6.1). Moreover, this mMoA provided a significantly better fit to the data (likelihood-ratio test, $p < 0.05$) than when assuming the same value of κ for all snails. This analysis indicates that the average value of κ differs between the animals in the control and in the lowest treatment. Whether this effect is caused by the low level of TBT cannot be confirmed from this data set (it would require a concentration-related change in the values of κ at several concentrations). Therefore, I did not incorporate the rather speculative effects on κ in my analysis with the complete data set for TBT (Chapter 4). This exercise, however, demonstrates that DEBkiss can deal quantitatively with particular non-monotonic concentration-responses, which are expected for endocrine-related effects in some cases. It would not be particularly demanding to incorporate this mMoA in future DEBkiss analyses when the data strongly suggest non-monotonic concentration-response patterns.

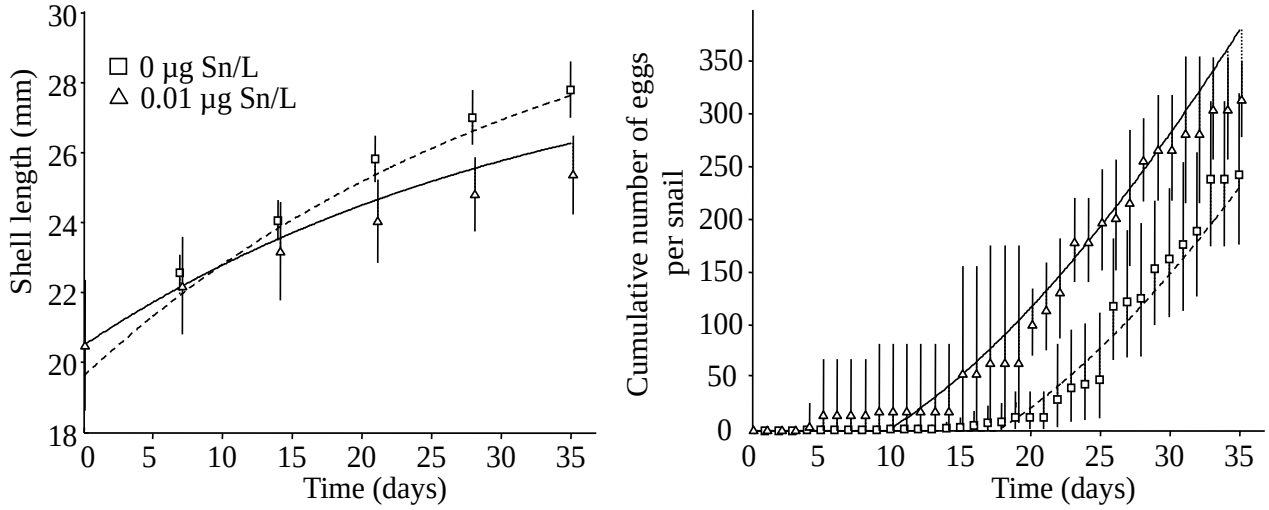

Figure 6.1. Response of *L. stagnalis* to the lowest TBT concentration (0.01 µg Sn/L). It is hypothesised that TBT induces a decrease of the fraction of assimilates allocated to soma (κ) for individuals exposed to the lowest concentration. Values for the parameter κ are kept constant during test duration and differed between control and exposed snails. A decrease of κ results in the increased cumulative number of eggs, but also in the decreased growth. Symbols represent the observed average values of snail responses, while bars are 95% confidence intervals. Curves are model fits to the data: broken lines for control and full lines for exposed snails. Other exposure concentrations are removed to facilitate readability. Complete test results are presented in Chapter 4.

Regarding putative endocrine disruptors, only the effects of TBT were analysed by using the DEBkiss model in my thesis. The data for the other compounds did not lend themselves for such an analysis due to their lack of effects on snails (Chapter 5), or the inappropriate test design for DEBkiss analysis of TPT data (Chapter 2 and Appendix 6). No clear indications for endocrine disruption were seen in the tests with TBT, although some subtle or specific effects (such as sperm counts and quality, or altered mating behaviour) might easily have gone unnoticed in my experiments because of the particular choice of endpoints. It still remains unclear whether existing

Table 6.1. Metabolic model parameters for *L. stagnalis* when assuming that TBT decreases the fraction of assimilates allocated to soma (κ) in snails exposed to 0.01 µg Sn/L.

Metabolic model parameters	Symbol	Value	Unit
Estimated values of metabolic parameters			
Specific maximum assimilation rate	J_{Am} [a]	0.250 (CI: 0.148-0.384)	mg/mm^2 d
Specific maintenance rate	J_M [v]	$1.61 \cdot 10^{-2}$ (CI: $8.61 \cdot 10^{-3}$-$2.69 \cdot 10^{-2}$)	mg/mm^3 d
Allocation fraction to soma (control)	κ_1	0.688 (CI: 0.634-0.772)	-
Allocation fraction to soma (0.01 µg Sn/L)	κ_2	0.613 (CI: 0.538-0.727)	-
Shell length at maturation (control)	L_{wp}	25.2 (CI: 24.4-25.9)	mm
Fixed values of metabolic parameters			
Blank hazard rate	h_0	$5.21 \cdot 10^{-20}$ (CI: $1 \cdot 10^{-20}$-$8.51 \cdot 10^{-4}$)	1/d
Shape correction coefficient	δ_M	0.369 (SE: $4.7 \cdot 10^{-2}$) [a]	-
Dry-weight density of structure	d_V	0.105 (SE: $1.6 \cdot 10^{-3}$) [a]	mg/mm^3
Dry weight of a freshly-laid egg	W_{B0}	0.13 (SE: $3 \cdot 10^{-3}$) [b]	mg
Yield of egg buffer on assimilates	y_{BA}	0.95 [c]	mg/mg
Yield of structure on assimilates	y_{VA}	0.8 [c]	mg/mg
Scaled functional response	f	1 [c]	-
Arrhenius temperature	T_A	8000 [d]	K

[a] Parameter values are not calibrated but fixed to the suggested values [24]; [b] Parameter value derived from the toxicity test with TBT; [c] Parameter values are not calibrated but fixed to the suggested values [22]; [d] The parameter value was not calibrated but fixed to the suggested value [35]. CI = 95% confidence interval; SE = standard error.

DEB-based models could deal with the specific effects of EDs. Such effects are not explicitly covered by DEB theory and might require additional modules.

6.4. Recommendations for test design

The most appropriate toxicity test design depends on the test purpose and on the envisaged type of data analysis. Based upon the experience I gained during my PhD project, I propose some improvements of test design for the OECD standardisation of reproduction tests with *L. stagnalis*. I also explain how toxicity test design for the DEBkiss analysis can be improved to match the modelling needs.

6.4.1. Recommendations for the improvement of the OECD reproduction test design with L. stagnalis

The proposed OECD test with *L. stagnalis* focuses mostly on adult reproduction [36]. As reproduction is regulated by the hormonal system, it is assumed that the effects of endocrine disruptors will be reflected on reproductive output. However, growth of snails is also regulated by the hormonal system and can be affected by endocrine disruptors. Effects of chemicals on growth of some invertebrates may strongly influence their populations [37]. The reason is that growth determines body size, and thereby feeding rates and the start of reproduction (which usually occurs at a constant body size). Specific effects on the growth process may be missed when initiating experiments with (nearly fully-grown) adults. As population sustainability is the protection goal of the ERA for aquatic invertebrates [38], focussing only on the reproduction effects may be insufficiently protective. Therefore, I would recommend monitoring growth of snails in toxicity tests. This is currently only an additional endpoint in the draft OECD protocol, but when adult snails are used, effects on growth may be easily missed. Effects on growth are best identified when tests are conducted with juvenile snails.

I have shown in this thesis that some chemicals decreased growth, which consequently affected reproduction. For the determination of the effects on growth, the shell length is a limited measure of body size. As the shell cannot decrease in size, shrinking of an animal's body will go unnoticed. Data on body dry weight provide a better quantitative relationship with toxicant effects, but this is of course a destructive measurement. As demonstrated in the Chapters 3 and 4, determination of the body dry weight at the end of a test (or when the individual dies) can provide a good indication of shrinking, in all age classes.

Shell length and dry weight of hatchlings were affected in the exposure test with 4-nonylphenol and adults, though at very high concentrations (Chapter 5). This indicates that the embryos were affected by the chemical itself or indirectly through the effects on mothers. Effects on hatching success, hatching time, and hatchling size, are important factors in population dynamics and thus there are good reasons to include these endpoints in the toxicity testing (although this is not done for other standard tests such as the *Daphnia* 21-d reproduction test). Following offspring performance after hatching might be an additional expansion of the test, to elucidate the potential for effects on the next generation (*e.g.*, [39, 40]).

Reproduction in standard toxicity tests is expressed as cumulated number of eggs. For the great pond snail, counting the number of clutches per snail (or taking the dry weight of clutches) can be used as a proxy for fecundity, and might be sufficient to detect effects of chemicals on snails. There is a good correlation of these endpoints with cumulated number of eggs per snail (Chapters 2-4), and some of the correlation coefficients are presented in Table 6.2. If reproducible, using this proxy might improve tests by reducing the most time-consuming task – the counting of the eggs. It could be envisaged that counting only a limited number of clutches per concentration per week would suffice to check that the correlation is valid, and then use this proxy to estimate the cumulated fecundity per individual (which is the core endpoint of the reproduction test).

Table 6.2. Correlation coefficients for cumulated fecundity and cumulated oviposition or cumulated dry weight of egg clutches. The data are results from the 35-d tests with sub-adults (Chapters 4 and 5, and Appendices 4 and 5).

	Chemical						
	TBT	TPT	MT	EE2	OP	NP	4nNP
Correlation coefficient for fecundity and oviposition	0.98	0.97	0.85	0.77	0.85	0.96	0.85
Correlation coefficient for fecundity and dry weight of egg clutches	0.99	0.99	0.94	0.91	0.97	0.98	0.91

To study potential ED effects, determining the proportion of abnormal eggs per clutch, and the different types of egg abnormalities as additional endpoints, might be required. Indeed, these endpoints were more sensitive than growth and fecundity in TBT tests where adults were exposed (Chapter 2). However, this higher sensitivity was not consistently observed in all tests; *e.g.* these endpoints were not more sensitive in TBT tests started with sub-adults (Chapter 4). Acetone also did not induce an increase of egg abnormalities at the concentrations I tested, although it has been shown to increase the proportion of polyembryonic eggs at higher tested concentrations [41]. Therefore, determining egg abnormalities as additional endpoint in toxicity tests should be considered.

Test duration of 21 d was sufficient to detect effects of relatively low concentrations of TBT (Chapter 2 and 4). To test low-concentration effects of some other chemicals, it may be advisable to expose animals for a longer time than 21 d. Charles and co-workers [42] have demonstrated that test design can be optimised in regard to the test duration and the number of replicates. For example, in a case study with cadmium and *L. stagnalis*, the authors showed that tests may last 35 d

with six replicates, and that three to four replicates would suffice for deriving descriptive statistics for test duration of 56 d.

For dissolving poorly soluble chemicals in test media, the OECD recommends using primarily physical methods. If chemicals cannot be dissolved by those methods, it is recommended to use solvents in concentrations up to 100 mg/L (0.1 ml/L or 0.01 % v.v.) [43], or even more restrictive up to 20 mg/L [44]. Based upon our result on toxicity tests with seven solvents (Appendix 7) we recommend the use of tri-ethylene glycol as it was the least harmful solvent for the great pond snail. Alternatively, acetone can be used.

6.4.2. Test design for data analysis with the DEBkiss model in L. stagnalis

The most valuable information from toxicity tests, with respect to DEB-based modelling, is data on survival, growth, and reproduction over time. For growth and reproduction, the DEBkiss model predicts the mass fluxes into these processes. Therefore, measuring experimental outputs in terms of dry mass would be preferable, as there is no need for conversions to the "currency" as followed in the model. Using more indirect measurements such as shell length and number of eggs adds uncertainty in the model analysis. Obtaining the dry body mass is a destructive measurement, which is not practical given the need for monitoring test endpoints over time (it would radically increase the number of test animals). Therefore, it makes sense to design an experiment such that some strategic information on dry weight can be obtained. Without using extra test animals, it is still possible to determine dry weight when the animal has died, or at the end of the test. Furthermore, some additional animals can be included in the test design to be sacrificed during the test, which was done in Chapters 3 and 4 to calibrate the model for control conditions. With these measurements, it is possible to establish the relationship between shell length and weight, and to test whether it changes across the treatments (which could signify shrinking).

Reproduction of the great pond snail can easily be determined by the dry mass of egg clutches (see Chapters 3-5). This is not only a more direct measurement for the parameterisation of the DEBkiss model, but also requires less experimental effort than counting eggs. However, it still makes sense to count the number of eggs in selected clutches as egg size may change with age of the mother or with the treatments. The investment per egg (and hence its size) is of particular relevance for population-level extrapolations, as it affects the performance of the offspring. Moreover, some toxicants may affect the number and size of eggs (*e.g.*, a decrease in size of eggs followed by an increase in the egg numbers per clutch) without modifying the total dry weigh of clutches from exposed animals (see [45]).

To calibrate the DEBkiss model for control conditions, it is clear from Chapter 3 and 4 that data on both sub-adult and adult life stages are of great importance. Data on adults only provide insufficient information to estimate the model parameters (the growth curve provides essential information). Data on sub-adults only provide sufficient information, but only if the test is continued sufficiently long to establish the reproduction rate. For a proper calibration of the DEBkiss model, data on both growth and reproduction are crucial, and they should be acquired preferably from the same group of animals. The fact that we had separate experiment with sub-adults (with insufficient reproduction data) and adults (with insufficient growth data) introduced additional uncertainty in the parameter estimation. Only when these data were tied together, parameters for the control conditions could be estimated. The best solution to this problem is to conduct a test with one cohort of animals, starting from juveniles that are not food limited (in the case of *L. stagnalis* fed with lettuce: shell length > 9 mm [46]). The test should then last long enough to provide an adequate amount of data on reproduction, at least not less than four weeks after the onset of oviposition, as suggested by Zimmer [47]. When tests are conducted with juveniles, they also provide the means to estimate the value of the parameter for size at maturation. Some chemicals affect this parameter (see [48]), so it is important to have a good estimate for it in control conditions.

In some cases, data on additional endpoints can be of great value to define the mMoA of the toxicant. For example, direct exposure of eggs to acetone turned out to be crucial to uniquely identify the mMoA of this chemical (Chapter 3). The ecological relevance of the effects on embryos was limited in this test setup, as the eggs were extracted from their gelatinous masses. Nevertheless, this test played an important role in elucidating the mMoA. Another endpoint that could be used to scrutinize the choice of mMoA is the respiration rate. This endpoint integrates all the energy fluxes that are dissipated from the metabolic machinery (maintenance costs, overhead costs for transformations). Hence, it can potentially be used to distinguish between mMoAs such as maintenance costs and assimilation, which have similar impacts on growth and reproduction. Also, establishing the feeding rate may be helpful in this respect, even if it is only done qualitatively (see Chapter 4 on TBT).

It has already been highlighted in Chapter 3 that DEBkiss analysis, and TKTD modelling in general [49], allows for flexibility in the toxicity test design. Exposure concentrations and test duration do not necessarily need to be constant and defined at the start of the test. For example, if too many test animals start dying, the exposure concentration can be switched to a lower one during the test. In Chapter 4 it has been shown that the model allows analysing data simultaneously from experiments performed at different temperatures and using different solvent concentrations. Furthermore, the DEBkiss model can accommodate variation of initial sizes of the test animals among the treatments (Chapters 3 and 4). This eases the constraint of ensuring "identical" sizes at the beginning of the test, and may thus reduce experimental costs. A DEB-based test design might allow the use of a smaller number of test animals, which also reduces experimental costs, but is also relevant for ethical reasons. However, the large variation between the individuals of the great pond snail is a matter of concern. The number of snails that I used in treatments (Chapters 3 and 4) was rather small, which hampers parameter identification. It is not clear if increasing the number of snails would help that much, as the average response of a highly variable group of individuals does not equal the response of the average animal, which may thus bias parameter estimation (see [50]).

6.5. Future directions

6.5.1. Accounting for inter-individual variability of test animals

In my experiments, I noticed that snail responses are very variable between individuals, and the data scatter is thus large. This variability can be attributed to the natural property of organisms to vary in their metabolism and physiology (either genotypically or phenotypically). Inter-individual variability of animals in toxicity tests may introduce a bias into data analysis and complicate the interpretation of toxic effects. A first attempt to deal with inter-individual variability in toxicity tests with TKTD modelling has been made in a case study with the springtail *Folsomia candida* exposed to cadmium [50]. By allowing some of the DEB model parameters to vary between individuals, life-cycle traits of each individual could be accurately described by the model [50]. This concept was further extended with the DEBkiss model in a part of my work on *L. stagnalis* ([51] and the conference poster, see www.alpar.debtox.info). However, the possibility to account for inter-individual differences in model parameters was severely hampered by the choice of test design. I followed individuals of sub-adult and adult snails separately in two experiments in Chapter 3. In retrospect, this was a poor choice, as none of the individuals had sufficient information on growth and reproduction to fit all model parameters. Following a single cohort of individuals over a longer time would have produced a far more useful data set to pursue the analysis of inter-individual differences.

Accounting for differences between individuals needs to be further developed. It would be beneficial for ecotoxicologists if this confounding factor in analyses could be overcome, especially for animals where the differences between individuals are large. A drawback from this type of DEB

analysis is that as the model accounts for each individual's energy budget, which makes the analysis extremely calculation-intensive and time-consuming.

6.5.2. Linking effects across the levels of biological organisation

Current methods for estimating and predicting chemical risks do not incorporate a mechanistic understanding of chemical effects. For predictive ecotoxicology it is crucial to understand qualitatively and quantitatively how chemical effects propagate across the levels of biological organisation [52]. Linking chemical exposure, starting from molecular events at sub-cellular level, with the effects on populations/communities/ecosystems has been conceptualised within the adverse outcome pathways (AOP) framework [53]. An AOP is defined as a "conceptual construct that portrays existing knowledge concerning the pathway of causal linkages between a molecular initiating event and a final adverse effect at a biological level of organisation that is relevant to a regulatory decision" [53] (Figure 6.2). The added value of the AOP approach is that it allows linking existing data conceptually and identifying data gaps that have to be filled in future studies.

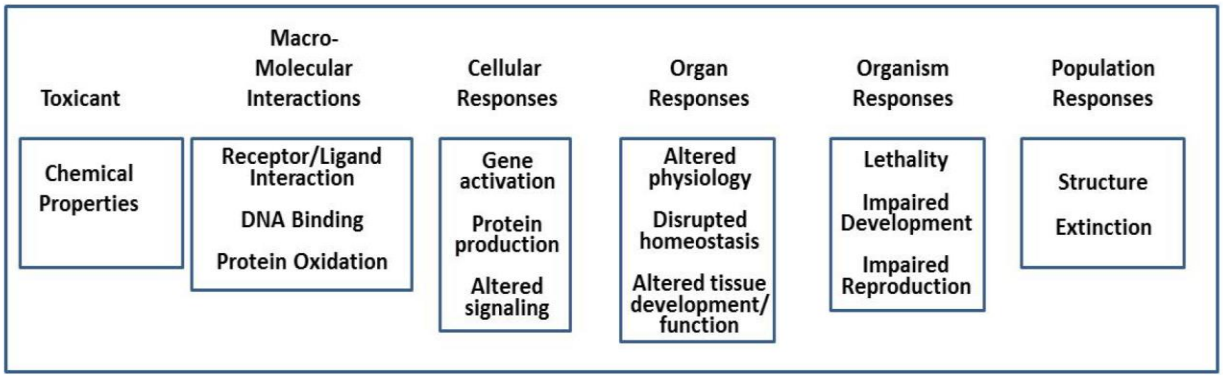

Figure 6.2. A schematic representation of the adverse outcome pathway framework (quoted from [54])

Regarding *L. stagnalis*, the piece of information we currently have is at the "omics" level and at the level of the whole individual (chemical effects on life-history traits); those are two possible starting points for developing AOPs for EDs in *L. stagnalis*. In my PhD thesis, I concentrated on responses of the whole individual because that is the level at which the most information is available. Since an individual is central in the AOP scheme, it allows investigating endocrine disruption with a top-down approach, as demonstrated in my DEBkiss analyses. Furthermore, gathered information about chemical impacts on apical test endpoints is suitable for estimating the population-level consequences of chemical exposure, which is indeed the goal of prospective ERA for aquatic invertebrates [38].

At the moment, mainly qualitative AOPs are being developed for a number of chemicals, including EDs. To make the best use of AOPs to forecast chemical impacts on populations, quantitative links across the levels of biological organisation must be established. Mechanistic models that explicitly connect each step in the propagation of toxic effects from sub-cellular to population levels can be used for establishing the links. Due to the (relative) complexity of such models, their use in building AOPs for regulatory purposes is still a long-term goal [55]. It is particularly difficult to develop models for processes at the molecular and cellular levels in invertebrates when there is little mechanistic information available.

Concerning not only EDs, but also other chemicals, TKTD models are well suited for bridging the gap between exposure and chemical effects at the population level (Figure 6.3), which can aid the development of quantitative AOPs. They provide insights into physiological processes that drive the effects, although with a huge simplification of the causalities at the lower levels of biological organisation. For example, it is not yet clear how the rather abstract DEB processes (assimilation, maintenance, growth *etc*.) relate to specific molecular interactions and cellular responses, and this relationship is likely to be species specific. To date, there have been only a few attempts in this

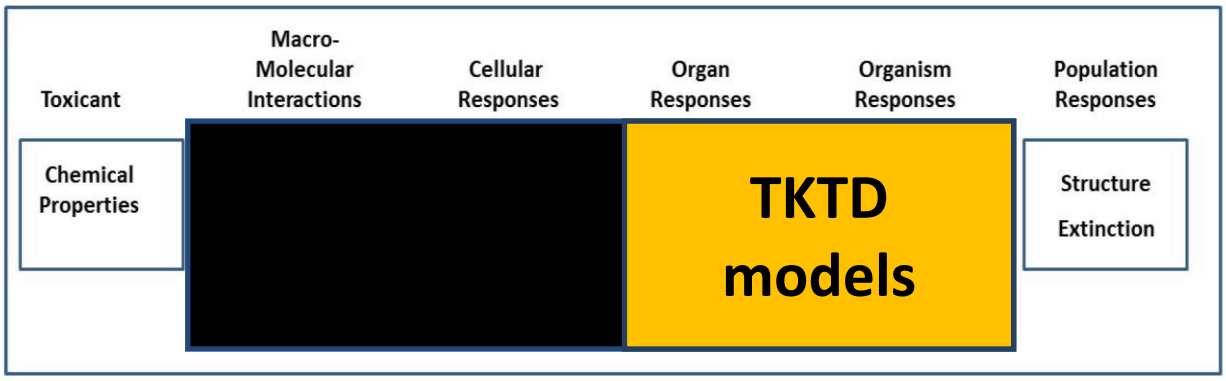

Figure 6.3. A schematic representation of where TKTD models belong in the adverse outcome pathway framework. In TKTD modelling, the description of exact interactions of toxicants with the molecular targets and cellular responses are still missing (black box).

direction (*e.g.*, [56, 57]), and thus it is still a major area for further investigations, particularly for EDs.

Combining experimental and TKTD modelling approaches can considerably improve current ERA practices by explaining why a chemical is toxic and under what circumstances, rather than only telling us how much of the chemical will affect *x* per cents of the test organisms at constant conditions [27]. Benefits of using TKTD models in ecotoxicology have been demonstrated in a number of studies (*e.g.*, [58, 59]), and they should now be much more applied for regulatory purposes. Overall, integration of experimental studies and mechanistic models to explore the effects of EDs and other chemicals across the levels of biological organisation is the way towards the incorporation of more ecology in the ERA of chemicals.

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Appendices

Appendix 1

Supporting Information for:

Considerations for test design to accommodate energy-budget models in ecotoxicology: a case study for acetone in the pond snail *Lymnaea stagnalis*

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Contents

1 Model description

DEBkiss is derived and presented in detail elsewhere [3]; here we restrict ourselves to presenting the actual model that is used in this study. The underlying model assumptions are summarised in Table 1, and the parameter symbols are collected in Table 2. Here, we immediately include the process of maturity maintenance, which improves the fit to reproduction data for the pond snail (see Supplemental Data of [3]), and provides a closer link to the well-established DEBtox model formulation [5].

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1. There are three types of biomass: food, assimilates and structural body components. Each type has a constant composition. They can be converted in each other with a certain constant efficiency. The state variables of the organism are the masses of the structural body, the reproduction buffer for adults, and the egg buffer used by the developing embryo. Total body mass is the sum of structure and reproduction buffer. The reproduction and egg buffer consist of assimilates.
2. The animal has three life stages: an embryo that does not feed but utilises the egg buffer, a juvenile that feeds but does not reproduce, and an adult that reproduces. The embryo starts with an egg buffer of assimilates and negligible structural mass. The first transition (birth) is triggered by the depletion of the egg buffer, and the second transition (puberty) by reaching a critical structural body weight.
3. The maximum feeding rate is proportional to the surface area of the animal. The animal is either searching for food or handling it (with constant handling time), leading to a hyperbolic functional response in the food density (Holling type II).
4. Food is instantly translated into assimilates that are directly used to fuel metabolic processes. Embryos assimilate their egg buffer at the maximum rate for their structural size (or a constant lower fraction of the maximum).
5. The flow of assimilates is split into a constant fraction κ for maintenance and structural growth (the soma), and $1 - \kappa$ for maturation and reproduction. From the κ flow, maintenance costs are paid first. Only structural biomass requires maintenance, which is proportional to its volume. The remainder of this flow is used for growth (with certain efficiency).
6. For adults, the $1 - \kappa$ flow is used to fill the reproduction buffer. For embryos and juveniles, all of the assimilates in this flux are burnt to increase complexity of the organism. At spawning events, the contents of the reproduction buffer are converted into eggs. The part of the buffer that was insufficient to create a single egg remains in the buffer. Transformation of buffer to egg comes with a certain (generally high) efficiency.
7. If feeding is insufficient to pay somatic maintenance costs, the organism first diverts energy from the 1-κ flux of assimilates and from the reproduction buffer. If that is insufficient, structure is converted into assimilates to pay maintenance (shrinking).
8. Maturity maintenance is proportional to structural volume for embryos and juveniles. After puberty, maturity maintenance is fixed to the level at puberty. From the $1-\kappa$ flux of assimilates, maturity maintenance is paid first (the remainder is used for maturation or reproduction). Under starvation, maturity maintenance is paid from the $1 - \kappa$ flux and the reproduction buffer as long as possible, but it is not paid from structure.

Table 1: The list of assumptions that leads to the DEBkiss model. The last assumption covers the extension for maturity maintenance

Symbol	Explanation	Dimension	Sugg. value
	Primary parameters		
f	Scaled functional response (0-1)	–	1
f_B	Apparent f for the embryo (0-1)	–	1
J^a_{Am}	Maximum area-specific assimilation rate	$m_a/(l^2t)$	–
J^v_J	Volume-specific maturity maintenance costs	$m_a/(l^3t)$	$J^v_M(1-\kappa)/\kappa$
J^v_M	Volume-specific somatic maintenance costs	$m_a/(l^3t)$	–
W_{B0}	Assimilates in a single freshly-laid egg	m_a	–
W_{Vp}	Structural body mass at puberty	m	–
y_{AV}	Yield of assimilates on structure (starvation)	m_a/m	0.8 mg/mg (dwt)
y_{BA}	Yield of egg buffer on assimilates	m_a/m_a	0.95 mg/mg (dwt)
y_{VA}	Yield of structure on assimilates (growth)	m/m_a	0.8 mg/mg (dwt)
κ	Fraction of assimilation flux for soma	–	0.8
	Conversions		
d_V	Dry-weight density of structure	m/l^3	0.1 mg/mm^3
δ_M	Shape correction coefficient	–	
	Fluxes, states and forcings		
J_A	Mass flux for assimilation	m_a/t	
J_H	Mass flux for maturation	m_a/t	
J_J	Mass flux for maturity maintenance	m_a/t	
J_M	Mass flux for somatic maintenance	m_a/t	
J_R	Mass flux to reproduction bufeer	m_a/t	
J_V	Mass flux for structure	m/t	
J_X	Mass flux of food	m_f/t	
W_B	Mass of assimilates buffer in egg	m_a	
W_R	Mass of reproduction buffer in adult	m_a	
W_V	Mass of structural body	m	
	Other output and secondary parameters		
L	Volumetric body length	l	
L_w	Physical body length	l	
R	Continuous reproduction rate	$\#/t$	
t_b	Hatching time for the egg	t	
W_{Vb}	Structural body mass at birth	m	

Table 2: Explanation of symbols, with dimensions given in mass (m for body, m_a for assimilates, and m_f for food), length (l_e for environment, l for organism), numbers (#), time (t). Suggested values for the yields (apart from y_{AV}) based on the typical values in [7].

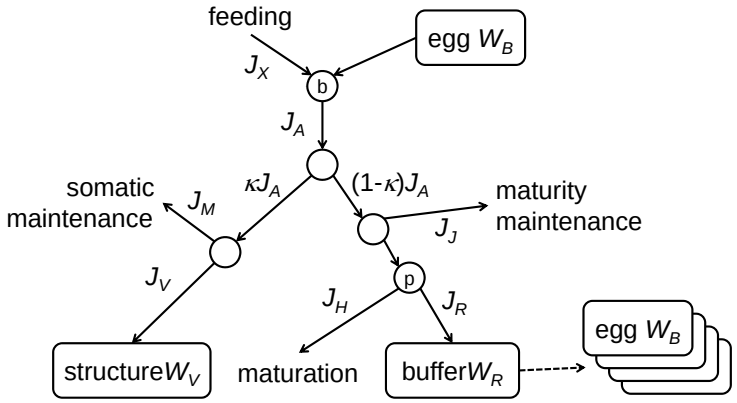

Figure 1: Schematic diagram of the mass flows in the DEBkiss model extended with maturity maintenance (and showing the maturation flux J_H and the feeding flux J_X, which are not further specified here). The node 'b' denotes a switch at birth (switching assimilation from the egg buffer to the result of feeding), and the node 'p' denotes a switch at puberty (switching investment into maturity to the reproduction buffer). Other nodes represent a continuous split of a mass flux.

1.1 Basic model

DEBkiss is a simplified version of the standard DEB animal model [8]. Most importantly, there is no distinction between structure and reserve (all body mass, apart from the reproduction or egg buffer is treated as structure), there is no state variable for maturity, and a different set of rules for embryonic development (needed due to the absence of a reserve compartment). The lack of a reserve compartment does not imply that there are no storages in the organism, but rather that these storages behave dynamically like structure (i.e., they require maintenance and cannot be used to fuel reproduction).

The basic model applies three state variable: the egg buffer W_B (used by the embryo), structural body mass W_V, and reproduction buffer W_R (see Fig. 1). Here, we only consider continuous reproduction (as adults of *L. stagnalis* regularly produce clutches of eggs), and we therefore do not use the reproduction buffer. The dynamics of the remaining state variables are given by:

$$\frac{d}{dt}W_B = -J_A \quad \text{until } W_B = 0, \text{ with } W_B(0) = W_{B0} \tag{1}$$

$$\frac{d}{dt}W_V = J_V \quad \text{with } W_V(0) \approx 0 \tag{2}$$

$$\tag{3}$$

Note that $t = 0$ here marks the start of development in the egg. The embryo will hatch when $W_B = 0$, which thereby determines the hatching time t_b and the dry weight at birth W_{Vb}. If we do not deal with embryos, we can start at any weight $W_{V0} > W_{Vb}$.

For some processes, we need to have access to the structural volume (L^3) of the animal. We can assume a constant density for structure (d_V):

$$L^3 = \frac{W_V}{d_V} \tag{4}$$

We can talk about L as the 'volumetric structural length' of the animal. If the structural biomass W_V is compressed into a cube, this will be the length of a side of that cube. In many cases, we measure body size of an animal as some length measure (e.g., the shell length

of snails). As long as the organism does not change in shape during growth, we can translate structural weight to some physical length (L_w) and vice versa using a constant correction factor δ_M:

$$L_w = \frac{L}{\delta_M} \tag{5}$$

The assimilates obtained from feeding are directly used in metabolism, and therefore, we do not consider any storage other than the reproduction buffer. The assimilation flux J_A is given by:

$$J_A = f J^a_{Am} L^2 \quad (\text{if } W_B > 0 \text{ then } f = 1 \text{ and } J^a_{Am} = f_B J^a_{Am}) \tag{6}$$

where f is the scaled functional response, which is the actual feeding rate at a certain food level divided by the maximum feeding rate for its current size. The scaled response f is thus between 0 (no food) and 1 (*ad libitum* food). Here, we treat f as a primary parameter, and do not consider the details of feeding, which is appropriate for constant or *ad libitum* food availability. For embryos, a different (lower) specific assimilation rate may be required, and hence the inclusion of a separate parameter f_B (see [3]).

Maintenance is the lump sum of all the processes needed to maintain the body's integrity. Assimilate buffers are assumed not to require maintenance, so the total maintenance flux is proportional to the structural body volume:

$$J_M = J^v_M L^3 \tag{7}$$

where J^v_M is the volume-specific maintenance rate coefficient. Maintenance costs are paid first, and the remaining flux to the soma is used for growth:

$$J_V = y_{VA}(\kappa J_A - J_M) \tag{8}$$

where y_{VA} is the yield of structural biomass on assimilates.

The maturity maintenance flux J_J is also proportional to the structural body volume, but only up to puberty:

$$J_J = J^v_J L^3 \quad \text{when } W_V < W_{Vp} \tag{9}$$

$$J_J = J^v_J L^3_p \quad \text{when } W_V \geq W_{Vp} \text{ (note: } L^3_p = W_{Vp}/d_V) \tag{10}$$

where J^v_J is the volume-specific costs for maturity maintenance. In principle, J^v_J is a primary parameter that can be fitted to experimental data. However, we will set it to a 'suggested value', by assuming a link with somatic maintenance:

$$J^v_J = \frac{1-\kappa}{\kappa} J^v_M \tag{11}$$

In the standard DEB model, linking these maintenance processes in this exact way yields the situation where the cumulative investment in maturity at puberty is independent of the food availability. This is one of the assumptions underlying the 'DEBtox' simplification [5]. The maturity maintenance flux is withdrawn from the $1-\kappa$ flux first, so the reproduction flux becomes:

$$J_R = (1-\kappa)J_A - J_J \quad (\text{if } W_V < W_{Vp} \text{ then } J_R = 0) \tag{12}$$

where W_{Vp} is the structural body mass where investment in reproduction starts (puberty). Note that W_{Vp} can easily be translated into a corresponding physical length L_{wp}.

Without reproduction buffer, the continuous reproduction rate R can be calculated as:

$$R = \frac{y_{BA} J_R}{W_{B0}} \tag{13}$$

where y_{BA} is the yield for the conversion of reproduction buffer to eggs, and W_{B0} is the dry weight of a single egg.

1.2 Starvation response

We need assumptions to deal with the situation of starvation, as varying food levels are common in the field, and because toxicants may affect the feeding or assimilation process. Starvation occurs when the allocated flux to the soma is insufficient to pay somatic maintenance ($\kappa J_A < J_M$). In the first stage, the total assimilation flux is still enough to pay both somatic and maturity maintenance (we ignore the reproduction buffer):

$$J_A > J_M + J_J: \quad J_V = 0 \text{ and for adults } J_R = J_A - J_M - J_J \tag{14}$$

In the second stage of starvation, the total assimilation flux is only enough for somatic maintenance (the maturity maintenance gets whatever is left):

$$J_M + J_J \geq J_A > J_M: \quad J_V = J_R = 0 \text{ and } J_J = J_A - J_M \tag{15}$$

In the third stage of starvation, the total assimilation flux is not enough for somatic maintenance, and the animal needs to shrink:

$$J_A \leq J_M: \quad J_V = (J_A - J_M)/y_{AV} \text{ and } J_R = J_J = 0 \tag{16}$$

where y_{AV} is the yield of assimilates (to pay maintenance) on structure. The maximum rates of feeding, assimilation and maintenance depend on structural size, so when the animal shrinks, these rates will decrease too.

1.3 Adding toxicokinetics

Symbol	Explanation	Dimension	Sugg. value
c_d	Dissolved concentration in water	m_q/l_e^3	–
c_V	Scaled concentration in structure	m_q/l_e^3	–
k_e	Elimination rate constant	$1/t$	–
k_e^*	Reference elimination rate constant	$1/t$	–
P_{RV}	Partition coeff. repro buffer-structure	m/m_a	1

Table 3: Explanation of additional symbols, with dimensions given in mass (m for body dwt., m_a for assimilates dwt., and m_q for chemical mass), length (l_e for environment, l for organism), time (t).

The simplest model for toxicokinetics (TK) is the first-order one-compartment model, where the entire organism is seen as a well-mixed homogeneous compartment. In the absence of a (considerable) reproduction buffer, we can use the following equation for the scaled (c_V) internal concentration in a growing organism (see [5]):

$$\frac{d}{dt} c_V = k_e^* \frac{L_m}{L} (c_d - c_V) - \frac{c_V}{W_V} \frac{d}{dt} W_V \tag{17}$$

where c_d is the dissolved concentration in water, and k_e^* is the reference elimination rate constant at maximum size in the control ($L_m = \kappa J_{Am}^a / J_M^v$). The elimination rate scales with a surface:volume and thus inversely with a length measure (as long as growth is isomorphic). The last term in the equation deals with growth dilution (and increase of the concentration when shrinking). Note that $c_V(\infty) = c_d$.

Chemical loss due to reproduction can easily be included in the model, when we assume that the concentration in the egg at egg laying is in equilibrium with the internal concentration of the mother. The chemical's affinity for the egg material is not necessarily the same as that for the adult tissues. Therefore, it is practical to introduce a partition coefficient between the egg material and the structure of the mother, P_{RV}. In practice, the value for P_{RV} will be unknown (it would require measurements of residues in mother and eggs), but we can depart from an equal affinity, and thus set $P_{RV} = 1$.

When we can consider reproduction to be continuous, for adults there will be a continuous flux of chemical out of the body with eggs. This flux can be subtracted from the changes in concentration as follows:

$$\frac{d}{dt}c_V = k_e^* \frac{L_m}{L}(c_d - c_V) - \frac{c_V}{W_V}\frac{d}{dt}W_V - \frac{W_{B0}R}{W_V}P_{RV}c_V \tag{18}$$

From this equation, it is obvious that the losses due to reproduction can be ignored by taking $P_{RV} = 0$.

For embryos, the surface:volume ratio (L_m/L) will be rather constant, as the size of the egg does not change much over the course of development. However, the elimination rate (k_e^*) will also be different, as it depends on the nature and shape of the interface. Further, the two forms of biomass in the egg (embryo structure and assimilate buffer) will change over time. Here, we chose to ignore these complications and assume instantaneous equilibrium between embryo tissue and the exposure medium: $c_V = c_d$.

1.4 Toxicant effects

Symbol	Explanation	Dimension	Sugg. value
b	Killing rate for survival	$l_e^3/(m_q t)$	–
c_0	Scaled no-effect concentration metabolic effects	m_q/l_e^3	–
c_{0s}	Scaled no-effect concentration survival	m_q/l_e^3	–
c_T	Tolerance concentration metabolic effects	m_q/l_e^3	–
h_Q	Hazard rate due to toxic stress	$1/t$	–
s	Stress factor for metabolic effects	$[-]$	–

Table 4: Explanation of additional symbols, with dimensions given in mass (m_q for chemical mass), length (l_e for environment), time (t).

The internal concentration can subsequently be linked to any of the primary parameters of the model (see [5, 4]). The affected parameter(s) is called the metabolic mode of action, or mMoA (see [1]). Following [6], we can use a linear-with threshold relationship for the dimensionless stress level on a parameter (in the control, $s = 0$):

$$s = \frac{1}{c_T}\max(0, c_V - c_0) \tag{19}$$

Here, the dose metric is the scaled internal concentration in structure c_V. The threshold or no-effect concentration is represented by c_0; below this NEC, the stress level will be zero. The

proportionality c_T is called the 'tolerance' concentration. Stress can increase or decrease the value of a parameter p like so:

$$p \to p(1+s) \quad \text{or} \quad p \to p\max(0, 1-s) \tag{20}$$

For some parameters there is room for discussion. Take the yield coefficient for structure on assimilates y_{VA}. A decrease in the yield can be implemented as $y_{VA}(1-s)$. But, if we interpret the effect as an increase in the overhead costs for growth, we should take $y_{VA}/(1+s)$. It all depends on our interpretation of effects. In the past, effects on yields have been implemented as an increase in the overheads [6, 4, 5].

Here, we selected an effect of acetone on the specific assimilation rate as follows (the superscript zero denotes the value in the control):

$$J_{Am}^a = J_{Am}^{a0}\max(0, 1-s) \tag{21}$$

this mode of action leaves open the possibility that the decrease in assimilation is caused by the feeding rate or the assimilation efficiency. For juveniles and adults, the net result under *ad libitum* food supply is the same; it does not matter whether less food is taken up or whether less energy is derived from the food. For embryos, however, it makes a big difference. A decrease on the feeding rate (the utilisation of the buffer in the egg) means that the assimilates that are not used are available to use at a later time. A decrease in the assimilation efficiency means that more of the egg buffer is lost in respiration, and thus not available for development.

Additionally, we tested an effect on both somatic and maturity maintenance:

$$J_M^v = J_M^{v0}(1+s) \tag{22}$$

Since the specific maturity maintenance is calculated from the somatic one (Eq. 11), this results in an effect on both maintenance processes.

Effects on survival can similarly be linked to the internal concentration, e.g., by using any of the toxicodynamic modules of the GUTS framework [2]. Here, we used the simple stochastic-death case, where the hazard rate due to toxic stress (h_Q) is given by:

$$h_Q = b\max(0, c_V - c_{0s}) \tag{23}$$

where c_{0s} is the threshold for effects on survival, and b is a proportionality constant referred to as the 'killing rate'. The translation from hazard rate to survival probability, and the associated statistical framework, is treated elsewhere [2].

1.5 Downloading the code

The Matlab code for the DEBkiss fit to the total data set (as presented in this paper) can be downloaded from `alpar.debtox.info`. The code runs on any Matlab platform and does not require additional toolboxes. Please note that this code is adapted to this specific analysis, so it cannot be directly used for other data sets. A more general version of this code is in preparation, and will be posted in the future to `www.debtox.info/debtoxm.php` (or a preliminary version can be requested by emailing Tjalling Jager at tjalling.jager@vu.nl). General DEBkiss calculations can also be performed with the BYOM code and DEBkiss package that can be found at `www.debtox.info/byom.php`.

2 Additional figures

2.1 Variability of snail traits in uncontaminated conditions

Figure 2 - 4 demonstrate the variability of the snails in the control treatment (in the absence of toxic stress).

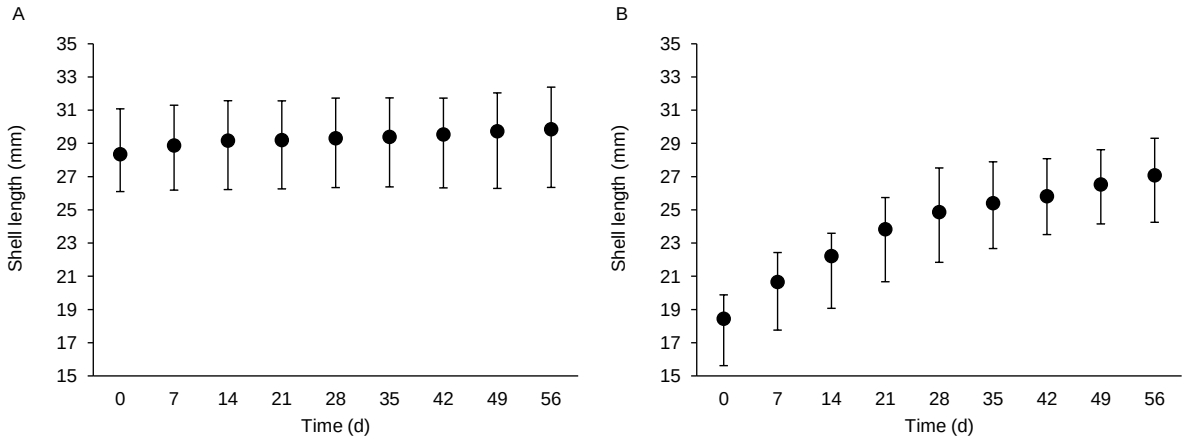

Figure 2: Shell lengths of snails in the control treatment. (A) adults, (B) juveniles. Error bars represent data range (minimum - maximum).

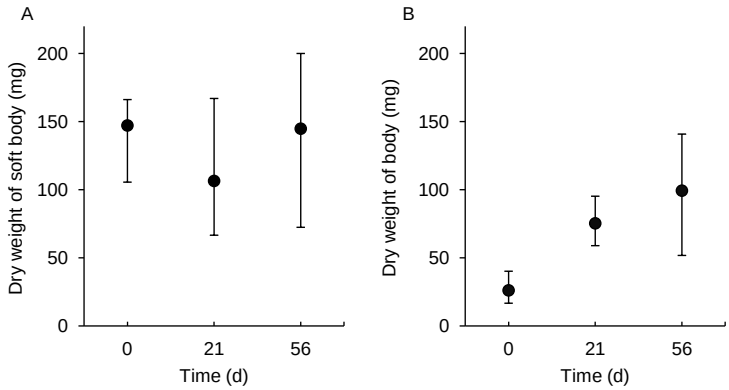

Figure 3: Dry weight of body of snails in the control treatment. (A) adults, (B) juveniles. Error bars represent data range (minimum - maximum).

2.2 Fitting data from recovery experiment

Model fits to effects of acetone on snails in the recovery test are shown in Figure 5. Snails were exposed for 14 days and then recovery was followed during 42 days. The model was calibrated on both exposure and recovery tests simultaneously (exposure tests shown in main text).

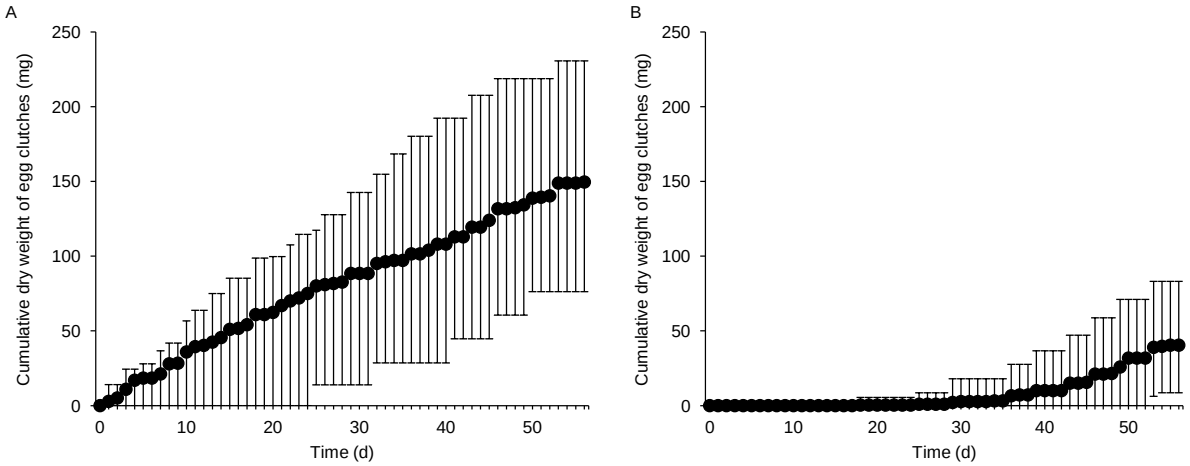

Figure 4: Cumulative dry weight of egg clutches in the control treatment. (A) adults, (B) juveniles. Error bars represent data range (minimum - maximum).

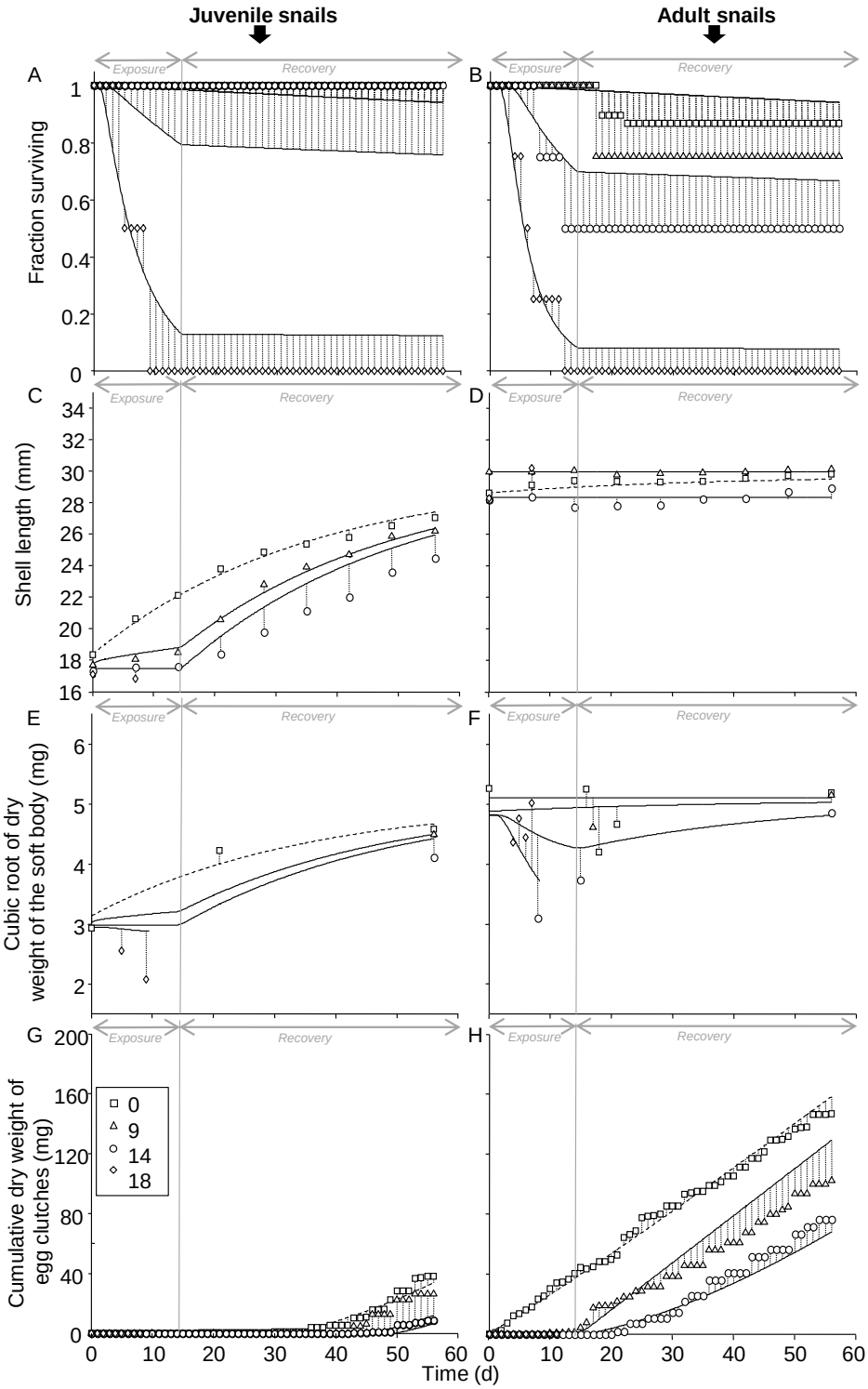

Figure 5: Model fits to effects of acetone on snails in the recovery test. The following endpoints are shown: (A,B) survival, (C,D) shell length, (E,F) dry weight of soft body, (G,H) cumulative dry weight of egg clutches. Lines represent model fits. Dotted line represents the model curve for control snails. Symbols represent experimental data from different exposure concentrations of acetone (ml/L).

2.3 Toxicological parameters from different calibration sets

Figure 6 is an extension of Figure 2 in the main text, and also includes calibration on the exposure-recovery data sets.

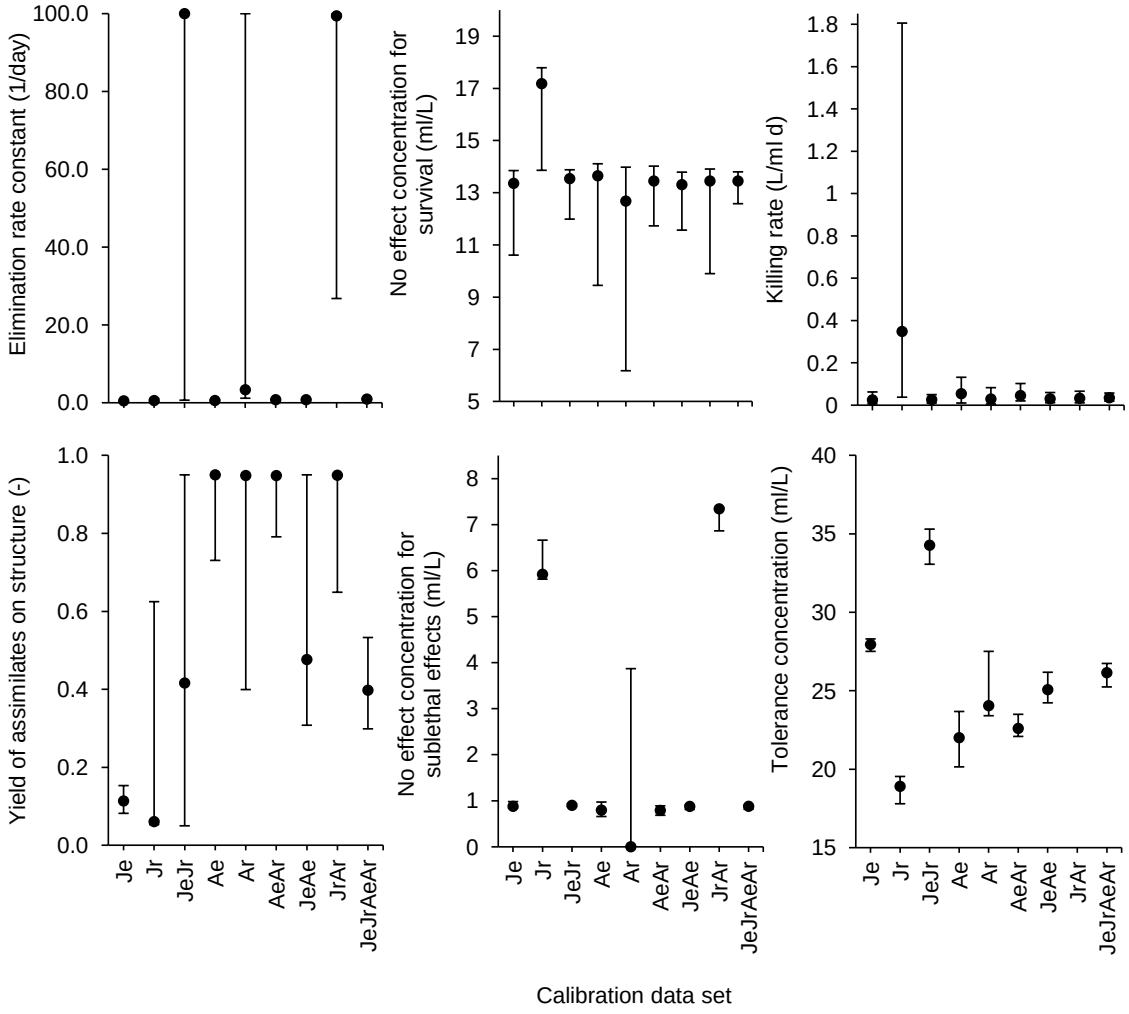

Figure 6: Toxicological model parameter values estimated from various calibration data sets. Error bars represent 95% confidence intervals.

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[1] O. Alda Álvarez, T. Jager, E. Marco Redondo, and J. E. Kammenga. Physiological modes of action of toxic chemicals in the nematode *Acrobeloides nanus*. *Environmental Toxicology and Chemistry*, 25:3230–3237, 2006.

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Appendix 2

Supporting Information for:

Integrated assessment of the effects of a putative endocrine disruptor tributyltin on *Lymnaea stagnalis* using the DEBkiss model

A. Barsi[a], T. Jager[b], L. Lagadic[a], G. Reljić[c], M. Collinet[a], V. Ducrot[a]

November 11, 2014

Contents

1 Model description

DEBkiss is derived and presented in detail elsewhere [4]. In this study, we use the model for ecotoxicological analyses in the pond snail as presented in Barsi et al. [2] (in their supplementary materials). Here, we largely repeat that model description, including the specific adaptations for this study:

1. A factor z to account for differences in bioavailability between the two experiments.
2. A Michaelis-Menten type of saturation in the toxicokinetics.
3. A toxicant effect on the feeding process.

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[c]Institute for Research on Generations and Family, Research Unit INSIDE, University of Luxembourg, Luxembourg

Symbol	Explanation	Dimension	Sugg. value
	Primary parameters		
f	Scaled functional response (0-1)	$-$	1
f_B	Apparent f for the embryo (0-1)	$-$	1
J^a_{Am}	Maximum area-specific assimilation rate	$m_a/(l^2t)$	$-$
J^v_J	Volume-specific maturity maintenance costs	$m_a/(l^3t)$	$J^v_M(1-\kappa)/\kappa$
J^v_M	Volume-specific somatic maintenance costs	$m_a/(l^3t)$	$-$
W_{B0}	Assimilates in a single freshly-laid egg	m_a	$-$
W_{Vp}	Structural body mass at puberty	m	$-$
y_{AV}	Yield of assimilates on structure (starvation)	m_a/m	0.8 mg/mg (dwt)
y_{BA}	Yield of egg buffer on assimilates	m_a/m_a	0.95 mg/mg (dwt)
y_{VA}	Yield of structure on assimilates (growth)	m/m_a	0.8 mg/mg (dwt)
κ	Fraction of assimilation flux for soma	$-$	0.8
	Conversions		
d_V	Dry-weight density of structure	m/l^3	0.1 mg/mm^3
δ_M	Shape correction coefficient	$-$	
	Fluxes, states and forcings		
J_A	Mass flux for assimilation	m_a/t	
J_H	Mass flux for maturation	m_a/t	
J_J	Mass flux for maturity maintenance	m_a/t	
J_M	Mass flux for somatic maintenance	m_a/t	
J_R	Mass flux to reproduction bufeer	m_a/t	
J_V	Mass flux for structure	m/t	
J_X	Mass flux of food	m_f/t	
W_B	Mass of assimilates buffer in egg	m_a	
W_R	Mass of reproduction buffer in adult	m_a	
W_V	Mass of structural body	m	
	Other output and secondary parameters		
L	Volumetric body length	l	
L_w	Physical body length	l	
R	Continuous reproduction rate	$\#/t$	
t_b	Hatching time for the egg	t	
W_{Vb}	Structural body mass at birth	m	

Table 1: Explanation of symbols for the basic model parameters, with dimensions given in mass (m for body, m_a for assimilates, and m_f for food), length (l_e for environment, l for organism), numbers (#), time (t). Suggested values for the yields (apart from y_{AV}) based on the typical values in [8].

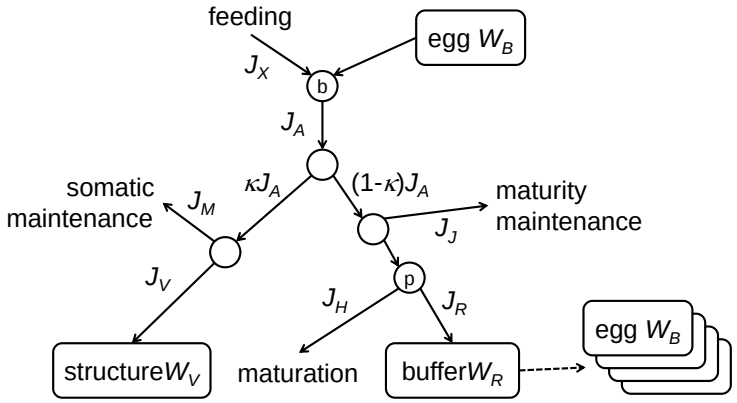

Figure 1: Schematic diagram of the mass flows in the DEBkiss model extended with maturity maintenance (and showing the maturation flux J_H and the feeding flux J_X, which are not further specified here). The node 'b' denotes a switch at birth (switching assimilation from the egg buffer to the result of feeding), and the node 'p' denotes a switch at puberty (switching investment into maturity to the reproduction buffer). Other nodes represent a continuous split of a mass flux.

1.1 Basic model

DEBkiss is a simplified version of the standard DEB animal model [9]. The basic model applies three state variable: the egg buffer W_B (used by the embryo), structural body mass W_V, and reproduction buffer W_R (see Fig. 1). Here, we only consider continuous reproduction (as adults of *L. stagnalis* regularly produce clutches of eggs), and we therefore do not use the reproduction buffer. The dynamics of the remaining state variables are given by:

$$\frac{d}{dt}W_B = -J_A \quad \text{until } W_B = 0, \text{ with } W_B(0) = W_{B0} \tag{1}$$

$$\frac{d}{dt}W_V = J_V \quad \text{with } W_V(0) \approx 0 \tag{2}$$

Note that $t = 0$ here marks the start of development in the egg. The embryo will hatch when $W_B = 0$, which thereby determines the hatching time t_b and the dry weight at birth W_{Vb}. If we do not deal with embryos, we can start at any weight $W_{V0} > W_{Vb}$.

Apart from body weight, we also need the structural volume (L^3) of the animal. We can assume a constant density for structure (d_V):

$$L^3 = \frac{W_V}{d_V} \tag{3}$$

We can talk about L as the 'volumetric structural length' of the animal. If the structural biomass W_V is compressed into a cube, this will be the length of a side of that cube. In many cases, we measure body size of an animal as some length measure (e.g., the shell length of snails). As long as the organism does not change in shape during growth, we can translate structural weight to some physical length (L_w) and vice versa using a constant correction factor δ_M:

$$L_w = \frac{L}{\delta_M} \tag{4}$$

The assimilates obtained from feeding are directly used in metabolism, and therefore, we do not consider any storage other than the reproduction buffer. The assimilation flux J_A is given by:

$$J_A = f J^a_{Am} L^2 \quad (\text{if } W_B > 0 \text{ then } f = 1 \text{ and } J^a_{Am} = f_B J^a_{Am}) \tag{5}$$

where f is the scaled functional response, which is the actual feeding rate at a certain food level divided by the maximum feeding rate for its current size. The scaled response f is thus between 0 (no food) and 1 (*ad libitum* food). Here, we treat f as a primary parameter, and do not consider the details of feeding, which is appropriate for constant or *ad libitum* food availability. For embryos, a different (lower) specific assimilation rate may be required, and hence the inclusion of a separate parameter f_B (see [4]).

Maintenance is the lump sum of all the processes needed to maintain the body's integrity. Assimilate buffers are assumed not to require maintenance, so the total maintenance flux is proportional to the structural body volume:

$$J_M = J^v_M L^3 \tag{6}$$

where J^v_M is the volume-specific maintenance rate coefficient. Maintenance costs are paid first, and the remaining flux to the soma is used for growth:

$$J_V = y_{VA}(\kappa J_A - J_M) \tag{7}$$

where y_{VA} is the yield of structural biomass on assimilates.

The maturity maintenance flux J_J is also proportional to the structural body volume, but only up to puberty:

$$J_J = J^v_J L^3 \quad \text{when } W_V < W_{Vp} \tag{8}$$

$$J_J = J^v_J L^3_p \quad \text{when } W_V \geq W_{Vp} \text{ (note: } L^3_p = W_{Vp}/d_V) \tag{9}$$

where J^v_J is the volume-specific costs for maturity maintenance. In principle, J^v_J is a primary parameter that can be fitted to experimental data. However, we will set it to a 'suggested value', by assuming a link with somatic maintenance:

$$J^v_J = \frac{1-\kappa}{\kappa} J^v_M \tag{10}$$

In the standard DEB model, linking these maintenance processes in this exact way yields the situation where the cumulative investment in maturity at puberty is independent of the food availability. This is one of the assumptions underlying the 'DEBtox' simplification [6]. The maturity maintenance flux is withdrawn from the $1-\kappa$ flux first, so the reproduction flux becomes:

$$J_R = (1-\kappa)J_A - J_J \quad (\text{if } W_V < W_{Vp} \text{ then } J_R = 0) \tag{11}$$

where W_{Vp} is the structural body mass where investment in reproduction starts (puberty). Note that W_{Vp} can easily be translated into a corresponding physical length L_{wp}.

Without reproduction buffer, the continuous reproduction rate R can be calculated as:

$$R = \frac{y_{BA} J_R}{W_{B0}} \tag{12}$$

where y_{BA} is the yield for the conversion of reproduction buffer to eggs, and W_{B0} is the dry weight of a single egg.

1.2 Starvation response

We need assumptions to deal with the situation of starvation, as varying food levels are common in the field, and because toxicants may affect the feeding or assimilation process. Starvation occurs when the allocated flux to the soma is insufficient to pay somatic maintenance ($\kappa J_A < J_M$). In the first stage, the total assimilation flux is still enough to pay both somatic and maturity maintenance (we ignore the reproduction buffer):

$$J_A > J_M + J_J : \quad J_V = 0 \text{ and for adults } J_R = J_A - J_M - J_J \tag{13}$$

In the second stage of starvation, the total assimilation flux is only enough for somatic maintenance (the maturity maintenance gets whatever is left):

$$J_M + J_J \geq J_A > J_M : \quad J_V = J_R = 0 \text{ and } J_J = J_A - J_M \tag{14}$$

In the third stage of starvation, the total assimilation flux is not enough for somatic maintenance, and the animal needs to shrink:

$$J_A \leq J_M : \quad J_V = (J_A - J_M)/y_{AV} \text{ and } J_R = J_J = 0 \tag{15}$$

where y_{AV} is the yield of assimilates (to pay maintenance) on structure. The maximum rates of feeding, assimilation and maintenance depend on structural size, so when the animal shrinks, these rates will decrease too.

1.3 Differences in temperature

We can assume that all rate constants (with a dimension that includes 'per time') scale in the same way with temperature. We can use the Arrhenius relationship to scale from a reference temperature T^* to the actual temperature T (both in Kelvin). All physiological rate constants have to be multiplied by:

$$F_T = \exp\left(\frac{T_A}{T^*} - \frac{T_A}{T}\right) \tag{16}$$

where T_A is the Arrhenius temperature in Kelvin. Lika and co-workers [8] suggest a value of 8000 K as typical value.

In our study, we also applied the same factor F_T to the elimination rate k_e and the killing rate b. Even though an effect of temperature on these parameters is to be expected, it is unclear whether the same value as for metabolic parameters applies.

1.4 Adding toxicokinetics

For the toxicity test of TBT with adults, we assume a slightly lower bioavailability. The bioavailability factor z is applied as follows:

$$c_d = z c_d \tag{17}$$

we added a Michaelis-Menten saturation on the uptake kinetics to account for the saturating effect patterns observed at higher exposures. This saturation was applied as a modification of the external concentration c_d:

$$c_d = \frac{c_d c_{dK}}{c_d + c_{dK}} \tag{18}$$

Symbol	Explanation	Dimension	Sugg. value
c_d	Dissolved concentration in water	m_q/l_e^3	–
c_{dK}	Half-saturation concentration	m_q/l_e^3	–
c_V	Scaled concentration in structure	m_q/l_e^3	–
k_e	Elimination rate constant	$1/t$	–
k_e^*	Reference elimination rate constant	$1/t$	–
P_{RV}	Partition coeff. repro buffer-structure	m/m_a	1
z	Bioavailability factor for adult test	$[-]$	–

Table 2: Explanation of additional symbols, with dimensions given in mass (m for body dwt., m_a for assimilates dwt., and m_q for chemical mass), length (l_e for environment, l for organism), time (t).

The simplest model for toxicokinetics (TK) is the first-order one-compartment model, where the entire organism is seen as a well-mixed homogeneous compartment. In the absence of a (considerable) reproduction buffer, we can use the following equation for the scaled (c_V) internal concentration in a growing organism (see [6]):

$$\frac{d}{dt}c_V = k_e^*\frac{L_m}{L}(c_d - c_V) - \frac{c_V}{W_V}\frac{d}{dt}W_V \tag{19}$$

where c_d is the dissolved concentration in water, and k_e^* is the reference elimination rate constant at maximum size in the control ($L_m = \kappa J_{Am}^a/J_M^v$). The elimination rate scales with a surface:volume and thus inversely with a length measure (as long as growth is isomorphic). The last term in the equation deals with growth dilution (and increase of the concentration when shrinking). Note that $c_V(\infty) = c_d$.

Chemical loss due to reproduction can easily be included in the model, when we assume that the concentration in the egg at egg laying is in equilibrium with the internal concentration of the mother. The chemical's affinity for the egg material is not necessarily the same as that for the adult tissues. Therefore, it is practical to introduce a partition coefficient between the egg material and the structure of the mother, P_{RV}. In practice, the value for P_{RV} will be unknown (it would require measurements of residues in mother and eggs), but we can depart from an equal affinity, and thus set $P_{RV} = 1$.

When we can consider reproduction to be continuous, for adults there will be a continuous flux of chemical out of the body with eggs. This flux can be subtracted from the changes in concentration as follows:

$$\frac{d}{dt}c_V = k_e^*\frac{L_m}{L}(c_d - c_V) - \frac{c_V}{W_V}\frac{d}{dt}W_V - \frac{W_{B0}R}{W_V}P_{RV}c_V \tag{20}$$

From this equation, it is obvious that the losses due to reproduction can be ignored by taking $P_{RV} = 0$.

1.5 Toxicant effects

The internal concentration can subsequently be linked to any of the primary parameters of the model (see [6, 5]). The affected parameter(s) is called the metabolic mode of action, or mMoA (see [1]). Following [7], we can use a linear-with threshold relationship for the dimensionless stress level on a parameter (in the control, $s = 0$):

$$s = \frac{1}{c_T}\max(0, c_V - c_0) \tag{21}$$

Symbol	Explanation	Dimension	Sugg. value
b	Killing rate for survival	$l_e^3/(m_q t)$	–
c_0	Scaled no-effect concentration metabolic effects	m_q/l_e^3	–
c_{0s}	Scaled no-effect concentration survival	m_q/l_e^3	–
c_T	Tolerance concentration metabolic effects	m_q/l_e^3	–
h_Q	Hazard rate due to toxic stress	$1/t$	–
s	Stress factor for metabolic effects	$[-]$	–

Table 3: Explanation of additional symbols, with dimensions given in mass (m_q for chemical mass), length (l_e for environment), time (t).

Here, the dose metric is the scaled internal concentration in structure c_V. The threshold or no-effect concentration is represented by c_0; below this NEC, the stress level will be zero. The proportionality c_T is called the 'tolerance' concentration. Stress can increase or decrease the value of a parameter p like so:

$$p \to p(1+s) \quad \text{or} \quad p \to p\max(0, 1-s) \tag{22}$$

For some parameters there is room for discussion. Take the yield coefficient for structure on assimilates y_{VA}. A decrease in the yield can be implemented as $y_{VA}(1-s)$. But, if we interpret the effect as an increase in the overhead costs for growth, we should take $y_{VA}/(1+s)$. It all depends on our interpretation of effects. In the past, effects on yields have been implemented as an increase in the overheads [7, 5, 6].

Here, we selected an effect of TBT on the scaled functional response as follows (the superscript zero denotes the value in the control), as this stress function provided the best explanation of the effect patterns:

$$f = \frac{f_0}{1+s} \tag{23}$$

This mode of action leaves open the possibility that the decrease in assimilation is caused by a decrease of the feeding rate (e.g., an increase in the handling time of food items) or a decrease of the assimilation efficiency (e.g., an increase in the overhead costs for assimilation). For juveniles and adults, the net result under *ad libitum* food supply is the same; it does not matter whether less food is taken up or whether less energy is derived from the food. For embryos, the details fo matter. A decrease on the feeding rate (the utilisation of the buffer in the egg) means that the assimilates that are not used are available to use at a later time. A decrease in the assimilation efficiency means that more of the egg buffer is lost in respiration, and thus not available for development.

Additionally, we tested an effect on both somatic and maturity maintenance:

$$J_M^v = J_M^{v0}(1+s) \tag{24}$$

Since the specific maturity maintenance is calculated from the somatic one (Eq. 10), this results in an effect on both maintenance processes.

Effects on survival can similarly be linked to the internal concentration, e.g., by using any of the toxicodynamic modules of the GUTS framework [3]. Here, we used the simple stochastic-death case, where the hazard rate due to toxic stress (h_Q) is given by:

$$h_Q = b\max(0, c_V - c_{0s}) \tag{25}$$

where c_{0s} is the threshold for effects on survival, and b is a proportionality constant referred to as the 'killing rate'. The translation from hazard rate to survival probability, and the associated statistical framework, is treated elsewhere [3].

1.6 Downloading the code

The Matlab code for the DEBkiss fit to the total data set (as presented in this paper) can be downloaded from alpar.debtox.info. The code runs on any Matlab platform and does not require additional toolboxes. Please note that this code is adapted to this specific analysis, so it cannot be directly used for other data sets. A more general version of this code is in preparation, and will be posted in the future to www.debtox.info/debtoxm.php (or a preliminary version can be requested by emailing Tjalling Jager at tjalling.jager@vu.nl). General DEBkiss calculations can also be performed with the BYOM code and DEBkiss package that can be found at www.debtox.info/byom.php.

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Appendix 3

Table 1. Frequency of observations

	Partial life-cycle tests			
	28d test with adults	56d test with adults	35d test with sub-adults	21d test with exposed offspring
	Treatments			
	4nNP Water and positive control (EE2)	NP Water, solvent, and positive control (EE2)	4nNP NP OP Water, solvent, and positive control (EE2)	EE2 Water control
Test endpoints for adults and sub-adults				
Survival	Daily	Daily	Daily	-
Shell length	Weekly	Biweekly	Weekly	-
Dry weight of soft body	-	Once [c]	-	-
Cumulated number of eggs per snail	Daily	-	Daily	-
Cumulated number of egg clutches per snail	Daily	Daily	Daily	-
Cumulated dry weight of egg clutches per snail	-	-	Daily	-
Number of eggs per clutch	Daily	-	Daily	-
Dry weight per egg clutch	-	Irregularly [d]	Daily	-
Dry weight per egg	-	-	Daily	-
Size at first oviposition	-	-	Daily	-
Age at first oviposition	-	-	Daily	-
Test endpoints for offspring originating from exposed parents				
Hatching success	Irregularly [a]	-	-	-
Time to hatch	-	Irregularly [e]	-	-
Dry weight of hatchlings	-	Once	-	-
Shell length of hatchlings	-	Once	-	-
Frequency of abnormal eggs	Irregularly [b]	-	Daily	-
Frequency of polyembryonic eggs	Irregularly [b]	-	Daily	-
Frequency of unfertilized eggs	Irregularly [b]	-	Daily	-
Frequency of albumen-atrophied eggs	Irregularly [b]	-	Daily	-
Test endpoints for offspring directly exposed to toxicants				
Hatching success	-	-	-	Once [f]
Time to hatch	-	-	-	Daily
Shell length of hatchlings	-	-	-	Once

[a] Hatching success was evaluated at the 35th day after oviposition, from clutches collected during the 4th week of the test; [b] the endpoint was evaluated from clutches collected during the 4th week of the test; [c] assessed at the end of the test only for snails exposed to NP; [d] assessed from clutches collected every seven days; [e] assessed from clutches collected on the following days: 9-40 and 50; [f] assessed at the day 21 of the test. "-" = the endpoint was not monitored.

Table 2. Statistical tests

	Partial life-cycle tests			
	28d test with adults	56d test with adults	35d test with sub-adults	21d test with exposed offspring
	Treatments			
	4nNP Water and positive control (EE2)	NP Water, solvent, and positive control (EE2)	4nNP NP OP Water, solvent, and positive control (EE2)	EE2 Water control
Test endpoints for adults and sub-adults				
Survival	*Jonckheere-Terpstra (JT)*	*JT*	*Cochran-Armitage*	-
Shell length	*JT*	*JT*	*Mann-Whitney with Bonferroni-Holm correction (MW-BH)*	-
Dry weight of soft body	-	*JT*	-	-
Cumulated number of eggs per snail	*JT*	-	*MW-BH*	-
Cumulated number of egg clutches per snail	*JT*	*JT*	*MW-BH*	-
Cumulated dry weight of egg clutches per snail	-	-	*MW-BH*	-
Number of eggs per clutch	*ANOVA* [a]	-	*JT* [d]	-
Dry weight per egg clutch	-	*JT* [b]	*JT* [d]	-
Dry weight per egg	-	-	*ANOVA* [d]	-
Size at first oviposition	-	-	*MW-BH*	-
Age at first oviposition	-	-	*MW-BH*	-
Test endpoints for non-exposed offspring				
Hatching success	*JT* [a]	-	-	-
Time to hatch	-	*ANOVA* [b]	-	-
Dry weight of hatchlings	-	+ [b, c]	-	-
Shell length of hatchlings	-	*ANOVA with Dunnett's test* [b]	-	-
Frequency of abnormal eggs	*MW-BH* [a]	-	*MW-BH* [d]	-
Frequency of polyembryonic eggs	*MW-BH* [a]	-	*MW-BH* [d]	-
Frequency of unfertilized eggs	*MW-BH* [a]	-	*MW-BH* [d]	-
Frequency of albumen-atrophied eggs	*MW-BH* [a]	-	*MW-BH* [d]	-
Test endpoints for exposed offspring				
Hatching success	-	-	-	*Cochran-Armitage*
Time to hatch	-	-	-	*JT*
Shell length of hatchlings	-	-	-	*JT*

[a] The sample size is shown in Table 3; [b] The sample size is shown in Table 4 of; [c] There was no replicated data, so a statistical test could not be performed; [d] The sample size is shown in Table 5.;

Table 3. Sample size for different endpoints in the 28-day test with adult snails.

	Partial life-cycle test 28d test with adults	
Test endpoints for adults and their offspring	**Treatments** EE2	4nNP
Number of eggs per clutch	$n_{CONTROL}$ = 108	
	$n_{EE2\ 0.0001}$ = 95	$n_{4nNP\ 0.0001}$ = 115
	$n_{EE2\ 0.001}$ = 110	$n_{4nNP\ 0.001}$ = 117
	$n_{EE2\ 0.01}$ = 125	$n_{4nNP\ 0.01}$ = 120
	$n_{EE2\ 0.1}$= 114	$n_{4nNP\ 0.1}$ = 130
	$n_{EE2\ 1}$ = 125	$n_{4nNP\ 1}$ = 114
	$n_{EE2\ 10}$ = 111	$n_{4nNP\ 10}$ = 131
Hatching success	$n_{CONTROL}$ = 11	
	$n_{EE2\ 0.0001}$ = 9	$n_{4nNP\ 0.0001}$ = 10
	$n_{EE2\ 0.001}$ =10	$n_{4nNP\ 0.001}$ = 10
	$n_{EE2\ 0.01}$ = 13	$n_{4nNP\ 0.01}$ = 9
	$n_{EE2\ 0.1}$= 8	$n_{4nNP\ 0.1}$ = 9
	$n_{EE2\ 1}$ = 14	$n_{4nNP\ 1}$ = 8
	$n_{EE2\ 10}$ = 10	$n_{4nNP\ 10}$ = 14
Frequency of abnormal eggs (including frequencies of the all abnormality types)	$n_{CONTROL}$ = 21	
	$n_{EE2\ 0.0001}$ = 18	$n_{4nNP\ 0.0001}$ = 22
	$n_{EE2\ 0.001}$ =24	$n_{4nNP\ 0.001}$ = 23
	$n_{EE2\ 0.01}$ = 28	$n_{4nNP\ 0.01}$ = 22
	$n_{EE2\ 0.1}$= 18	$n_{4nNP\ 0.1}$ = 22
	$n_{EE2\ 1}$ = 28	$n_{4nNP\ 1}$ = 24
	$n_{EE2\ 10}$ = 22	$n_{4nNP\ 10}$ = 28

$n_{X\ Y}$ = number of clutches "n" in the exposure to the compound "$_X$" and concentration "$_Y$" (µg/L).

Table 4. Sample size for different endpoints in the 56-day test with adult snails.

	Partial life-cycle test 56d test with adults	
Test endpoints for adults and their offspring	**Treatments** EE2	NP
Dry weight per egg clutch	$n_{CONTROL}$ = 95	
	$n_{EE2\ 0.01}$ = 37	$n_{NP\ 0.1}$ = 20
	$n_{EE2\ 0.055}$ = 34	$n_{NP\ 0.55}$ = 37
	$n_{EE2\ 0.3}$ = 43	$n_{NP\ 3}$ = 27
	$n_{EE2\ 1.7}$ = 40	$n_{NP\ 17}$ = 22
	$n_{EE2\ 9.1}$ = 28	$n_{NP\ 91}$ = 21
	$n_{EE2\ 50}$ = 27	$n_{NP\ 500}$ = 19
Time to hatch	$n_{CONTROL}$ = 185	
	$n_{EE2\ 0.01}$ = 83	$n_{NP\ 0.1}$ = 80
	$n_{EE2\ 0.055}$ = 71	$n_{NP\ 0.55}$ = 87
	$n_{EE2\ 0.3}$ = 76	$n_{NP\ 3}$ = 81
	$n_{EE2\ 1.7}$ = 65	$n_{NP\ 17}$ = 86
	$n_{EE2\ 9.1}$ = 72	$n_{NP\ 91}$ = 79
	$n_{EE2\ 50}$ = 60	$n_{NP\ 500}$ = 65
Dry weight and shell length of hatchlings.	Number of clutches from which hatchlings were collected:	
	$n_{CONTROL}$ = 13	
To obtain the mean dry weight, hatchlings from one exposure were pooled and the total mass was divided by n. *Therefore, the data on dry weight were not suitable for a statistical analysis.*	$n_{EE2\ 0.055}$ = 4	$n_{NP\ 0.55}$ = 5
	$n_{EE2\ 1.7}$ = 5	$n_{NP\ 17}$ = 6
	$n_{EE2\ 50}$ = 6	$n_{NP\ 500}$ = 4
	Number of hatchlings:	
	$n_{CONTROL}$ = 81	
	$n_{EE2\ 0.055}$ = 32	$n_{NP\ 0.55}$ =36
	$n_{EE2\ 1.7}$ = 31	$n_{NP\ 17}$ = 33
	$n_{EE2\ 50}$ = 30	$n_{NP\ 500}$ = 30

$n_{X\ Y}$ – number of clutches or of hatchlings "n" in the exposure to the compound "$_X$" and the concentration "$_Y$" (µg/L).

Table 5. Sample size for different endpoints in the 35-day test with sub-adult snails.

	Partial life-cycle test 35d test with sub-adults			
Test endpoints for sub-adults and their offspring	**Treatments** EE2	OP	NP	4nNP
(i) Number of eggs per clutch	$n_{CONTROL}$ = 62			
	$n_{EE2\ 0.01}$ = 8	$n_{OP\ 0.05}$ = 4	$n_{NP\ 0.05}$ = 13	$n_{4nNP\ 0.05}$ = 10
(ii) Dry weight per egg clutch	$n_{EE2\ 0.05}$ = 16	$n_{OP\ 0.25}$ = 18	$n_{NP\ 0.25}$ = 8	$n_{4nNP\ 0.25}$ = 14
	$n_{EE2\ 0.25}$ = 13	$n_{OP\ 1.25}$ = 7	$n_{NP\ 1.25}$ = 7	$n_{4nNP\ 1.25}$ = 16
(iii) Dry weight per egg	$n_{EE2\ 0.6}$ = 19	$n_{OP\ 6.25}$ = 23	$n_{NP\ 6.25}$ = 15	$n_{4nNP\ 6.25}$ = 15
	$n_{EE2\ 1}$ = 14	$n_{OP\ 15}$ = 14	$n_{NP\ 15}$ = 12	$n_{4nNP\ 15}$ = 13
(iv) Frequency of abnormal eggs (including frequencies of the all abnormality types)	$n_{EE2\ 5}$ = 16	$n_{OP\ 36}$ = 12	$n_{NP\ 36}$ = 11	$n_{4nNP\ 36}$ = 17
	$n_{EE2\ 10}$ = 12	$n_{OP\ 86}$ = 7	$n_{NP\ 86}$ = 12	$n_{4nNP\ 86}$ = 7
	$n_{EE2\ 20}$ = 10	$n_{OP\ 200}$ = 13	$n_{NP\ 200}$ = 8	$n_{4nNP\ 200}$ = 7
	$n_{EE2\ 40}$ = 17	$n_{OP\ 500}$ = 10	$n_{NP\ 500}$ = 1	$n_{4nNP\ 500}$ = 12
	$n_{EE2\ 80}$ = 14	$n_{OP\ 1000}$ = 2	$n_{NP\ 1000}$ = 0	$n_{4nNP\ 1000}$ = 15

$n_{X\ Y}$ = number of clutches "n" in the exposure to the compound "$_X$" and concentration "$_Y$" (µg/L).

Appendix 4

Test results on exposure of Lymnaea stagnalis to ethynyloestradiol and alkylphenols 4-nonylphenol, 4-n-nonylphenol, and 4-tert-octylphenol

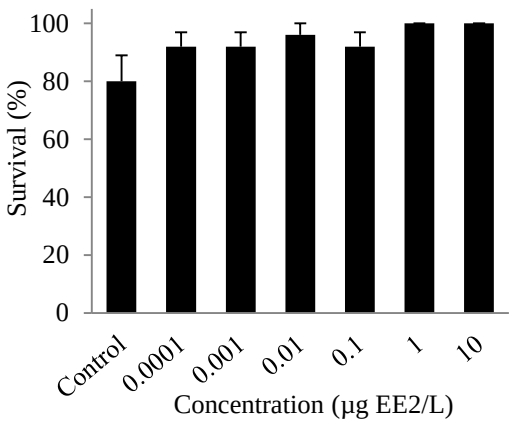
In Chapter 5, I showed results of partial life-cycle toxicity tests in which *Lymnaea stagnalis* was exposed to ethynyloestradiol (EE2), 4-nonylphenol (NP), 4-n-nonylphenol (4nNP), and 4-tert-octylphenol (OP). In this appendix, I present all results collected from all above-mentioned tests, because I believe they can be useful for future research development in the domain of endocrine disruption in aquatic gastropods.

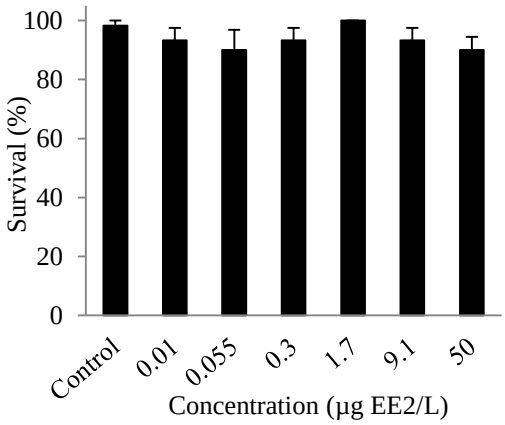
Design and statistical analysis of the tests are described in chapter 5 and in the corresponding appendices. Just as a reminder, all data are presented as mean values and their standard errors, unless stated otherwise. Highly significant probability of the test statistic, $0.001 < p < 0.01$ and $0.0001 < p < 0.001$, was highlighted on figures by two and three asterisks, respectively. The term "control" on the graphs refers to pooled data from clean water control and solvent control. Where data between these two types of controls were statistically significant, they are shown separately.

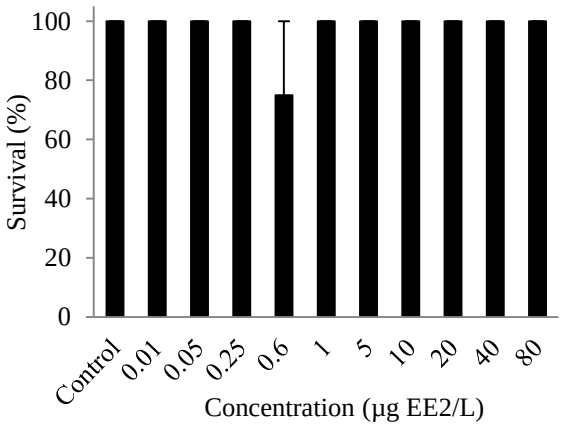
1. Ethynyloestradiol

1.1 Adults and sub-adults

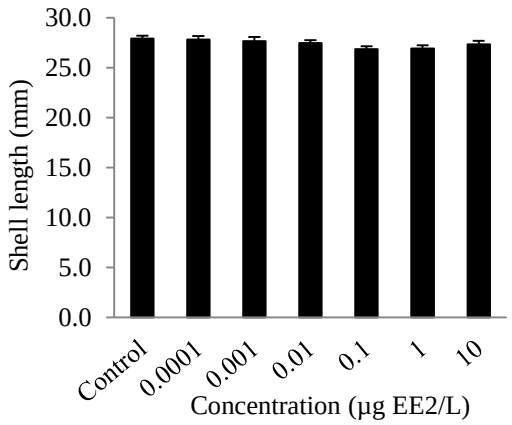
Survival

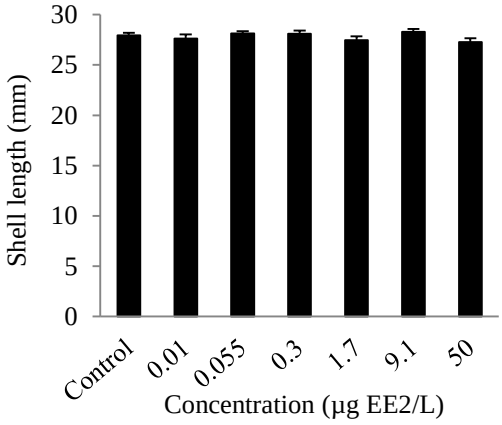


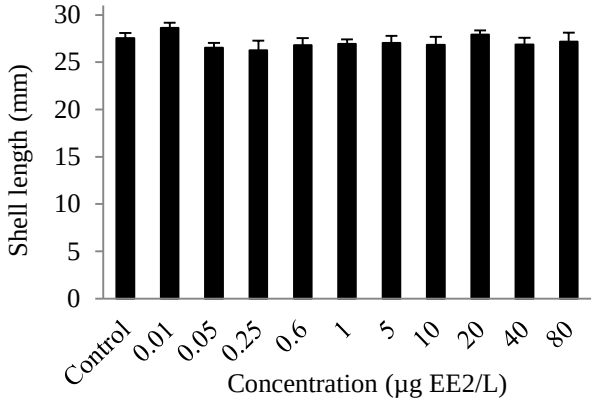




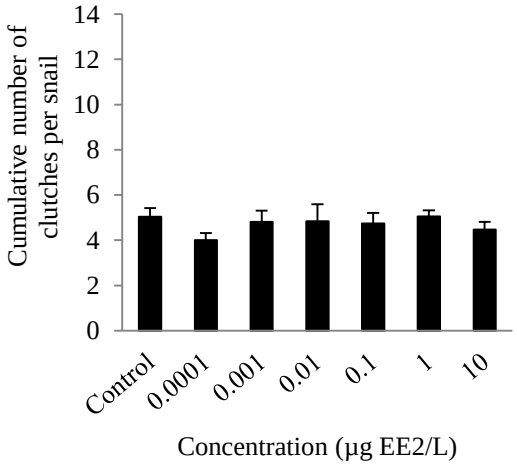
Shell length

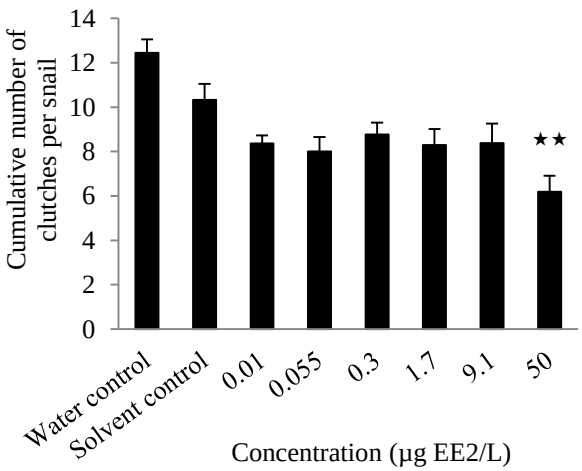


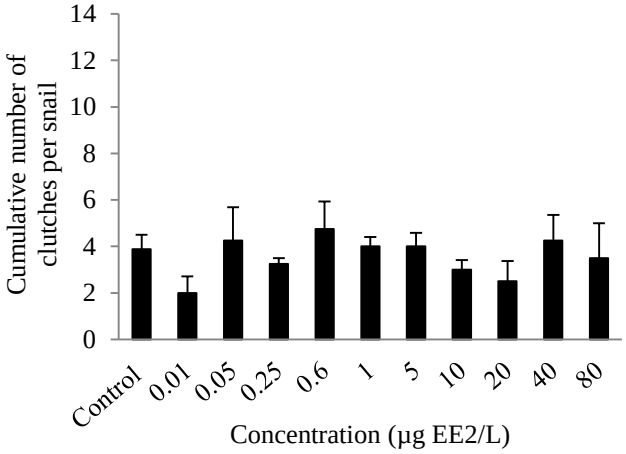




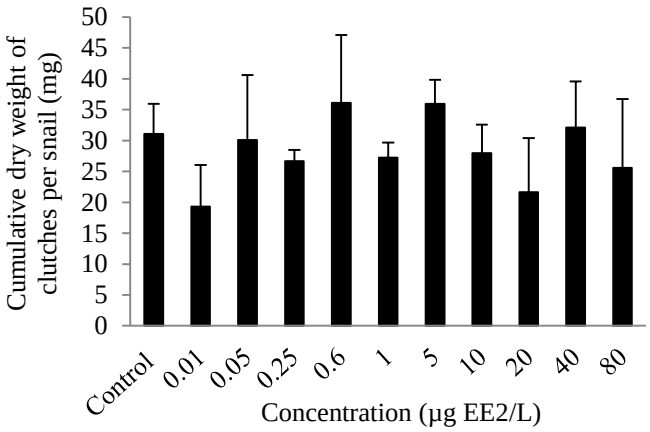
Cumulative number of clutches



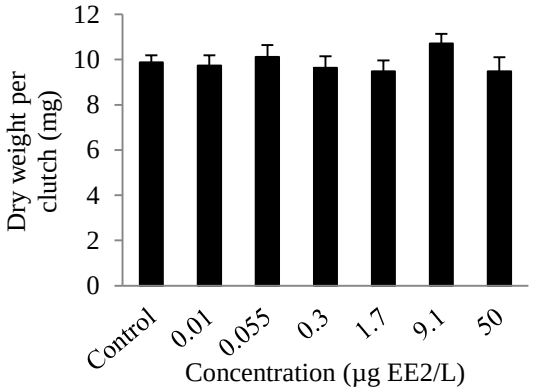


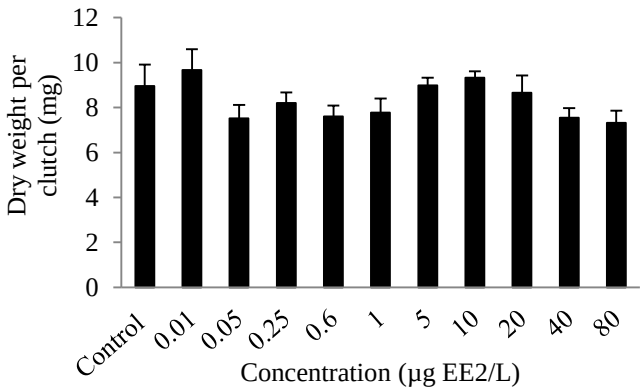


Cumulative dry weight of clutches

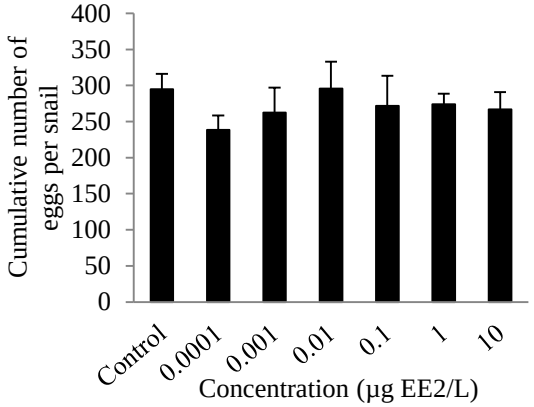


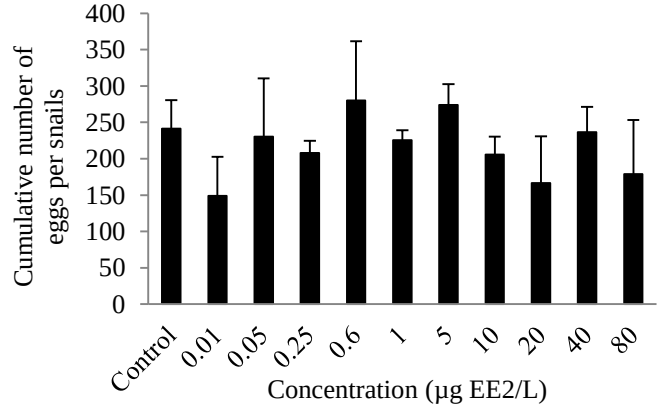
Dry weight per clutch



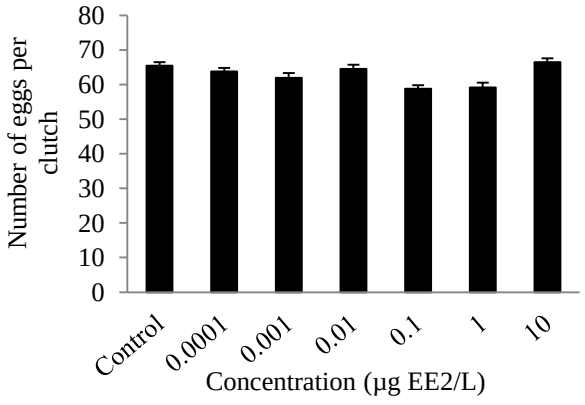


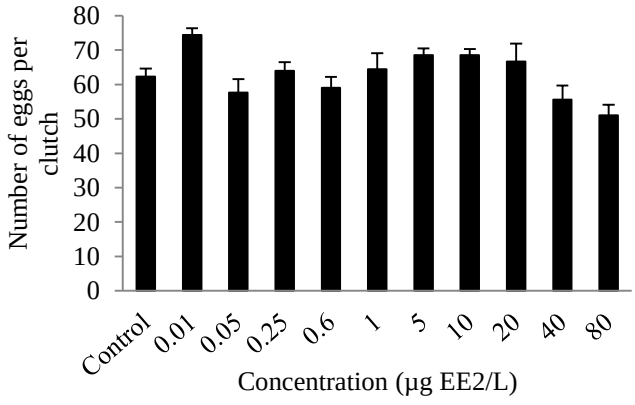
Cumulative number of eggs



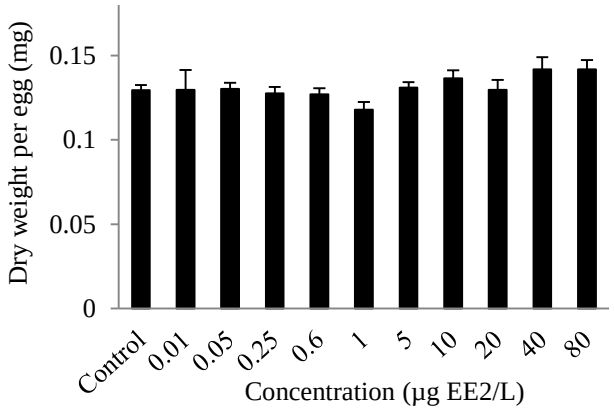


Number of eggs per clutch

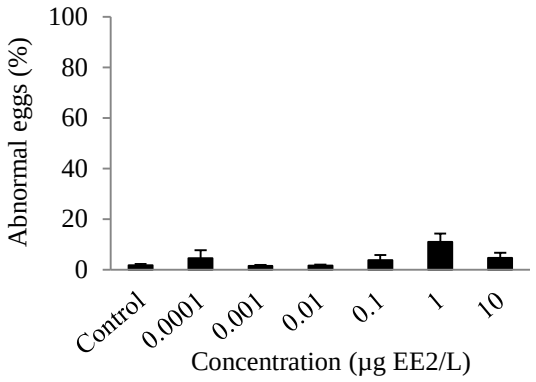


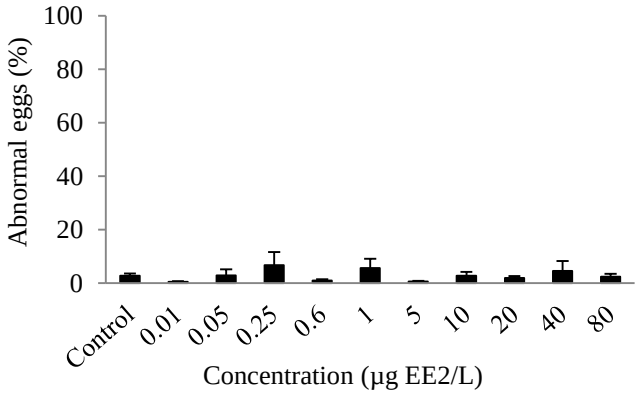


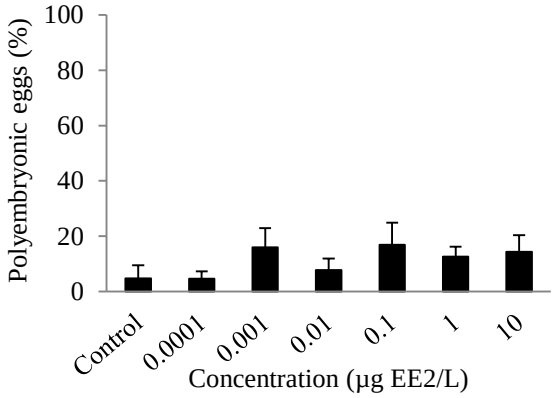
Dry weight per egg

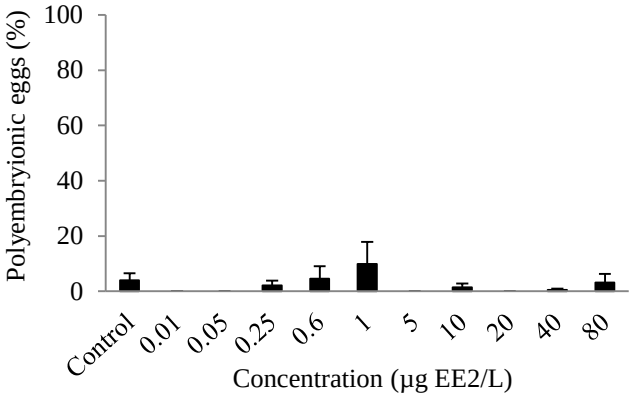


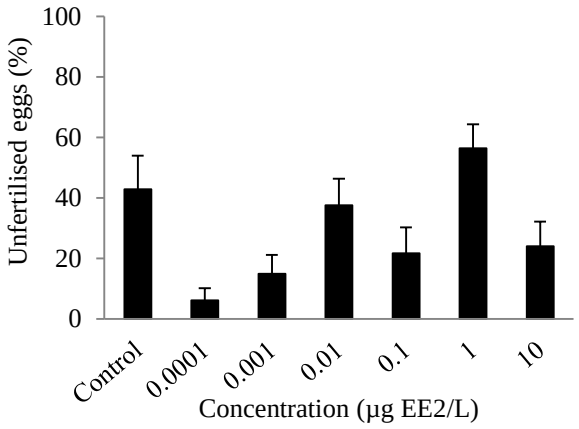
Egg abnormalities

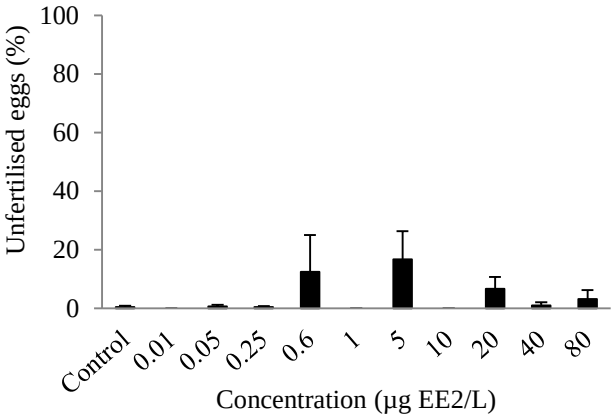


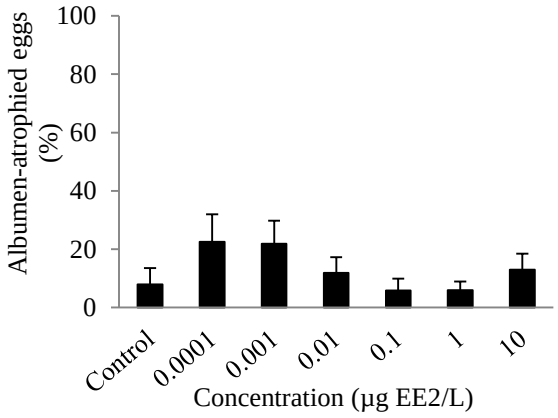


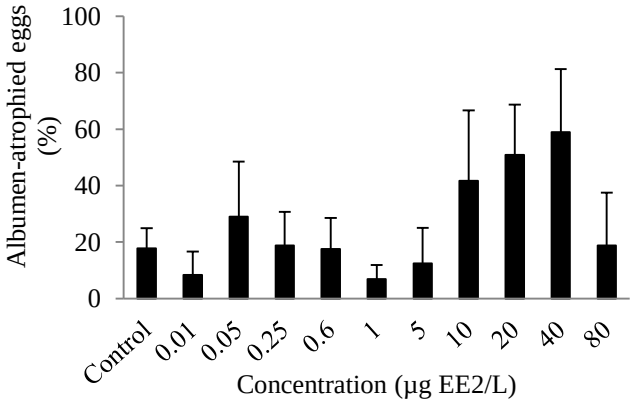




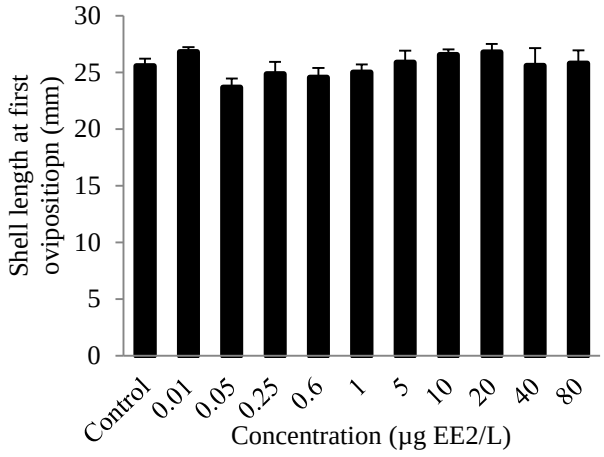


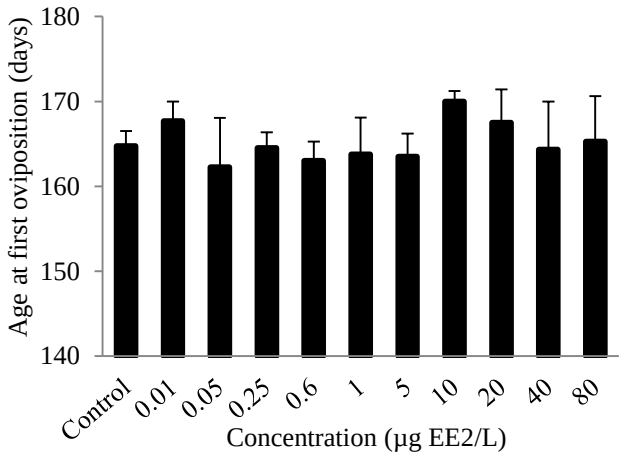






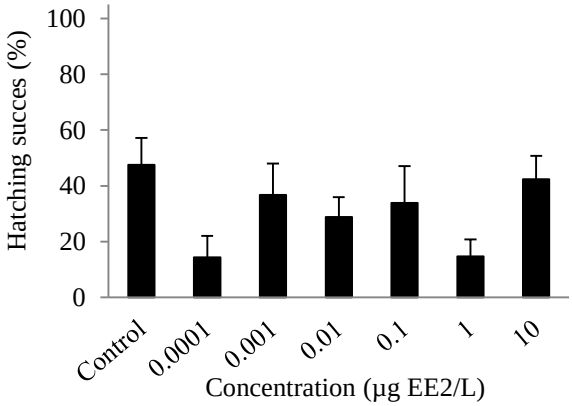
Shell length and age at first oviposition



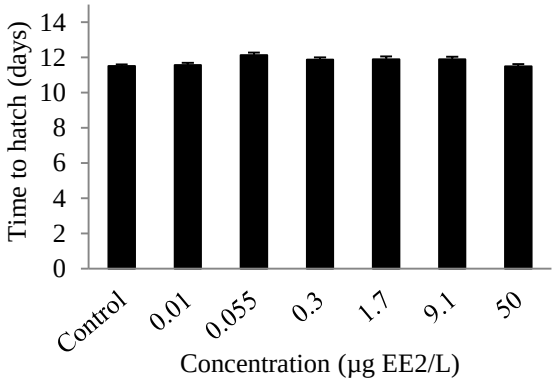


1.2. Non-exposed offspring of treated snails

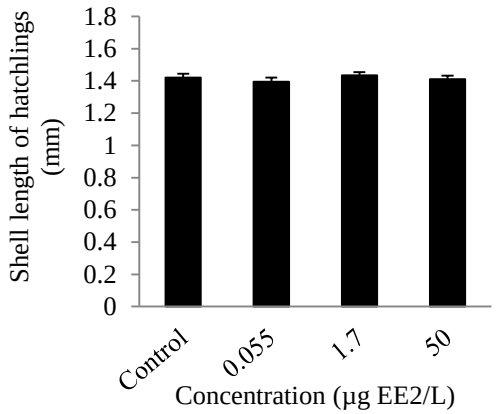
Hatching success. Clutches were collected during 4th week of the test. Hatching success was evaluated after a 35 days. During this time clutches stayed in clean water.

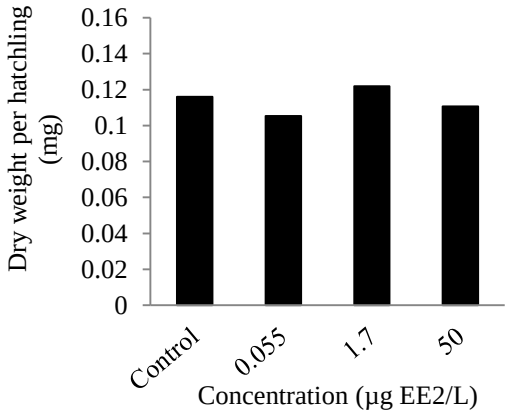


Time to hatch (in clean water)



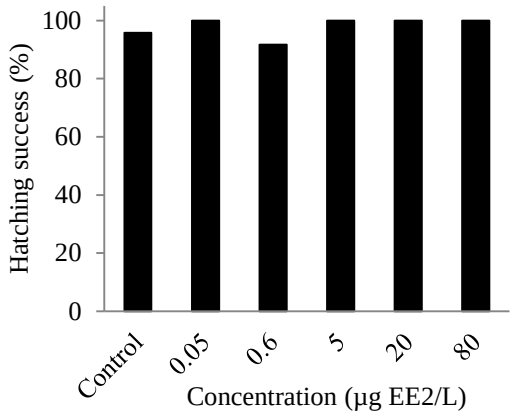
Shell length and dry weight of hatchlings. Clutches were collected on 54th day of the test.

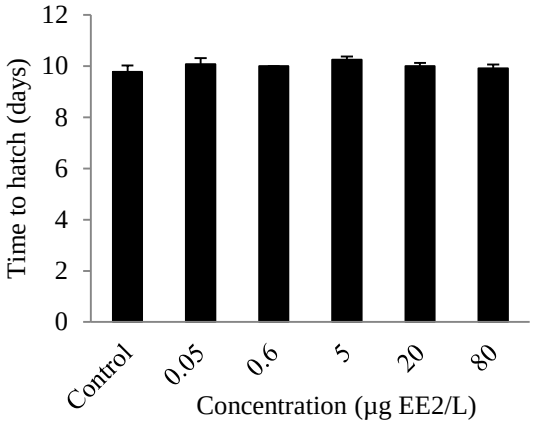


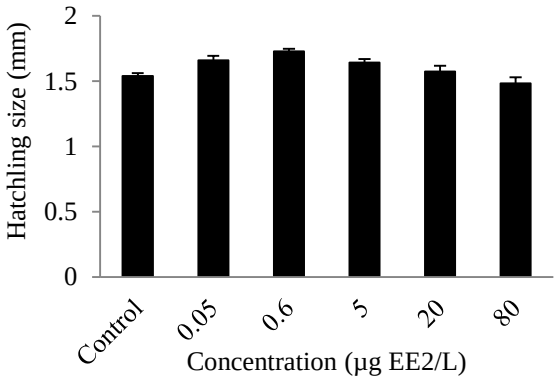


1.3. Exposed offspring of non-exposed snails.

Hatching success, time to hatch, and hatchling size. Egg clutches were collected from non-exposed snails in laboratory culture. Then eggs were taken out from jelly-masses, each egg was placed into a plastic well, and directly exposed to EE2 in isolation during 21 days.







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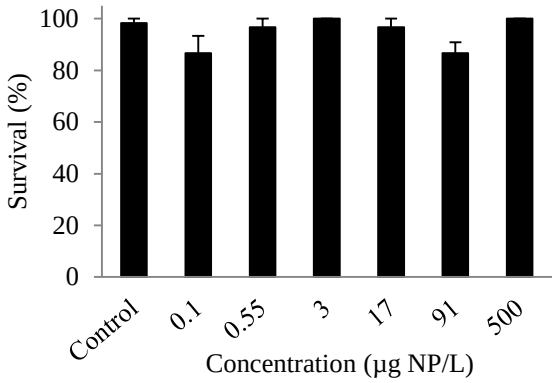
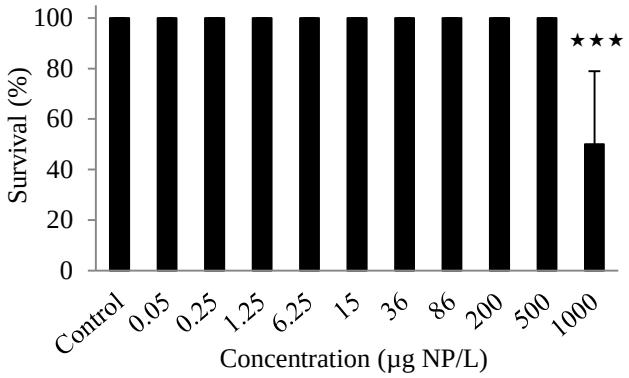
2. 4-Nonylphenol (NP)

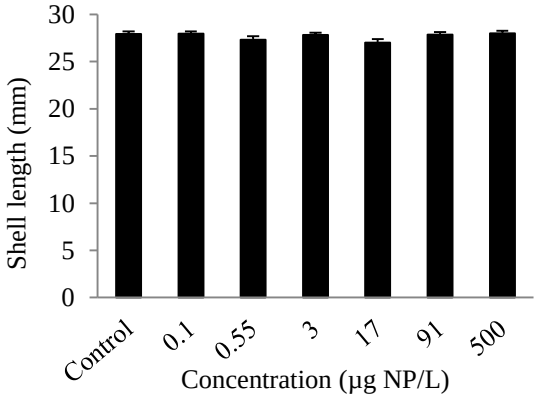
2.1 Adults and sub-adults

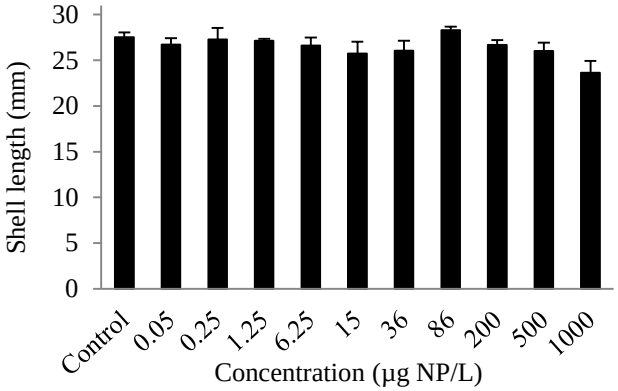
Survival



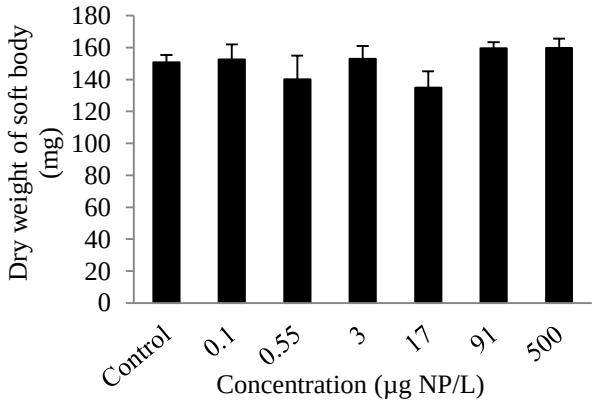


Shell length

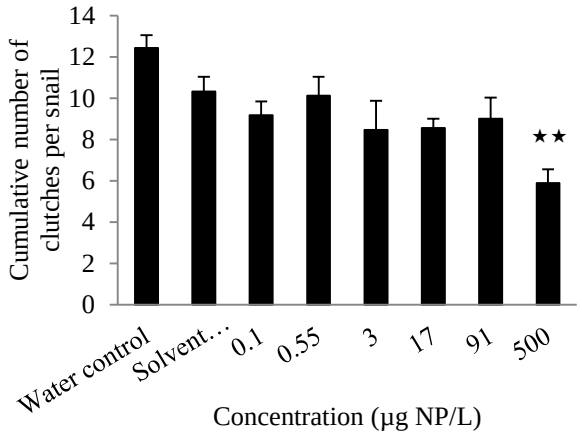


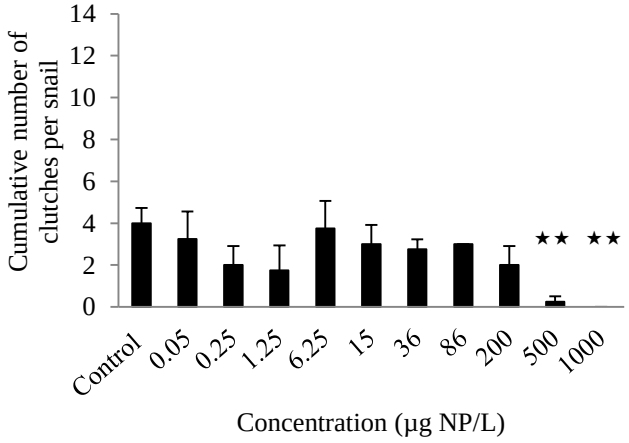


Dry weight of soft body

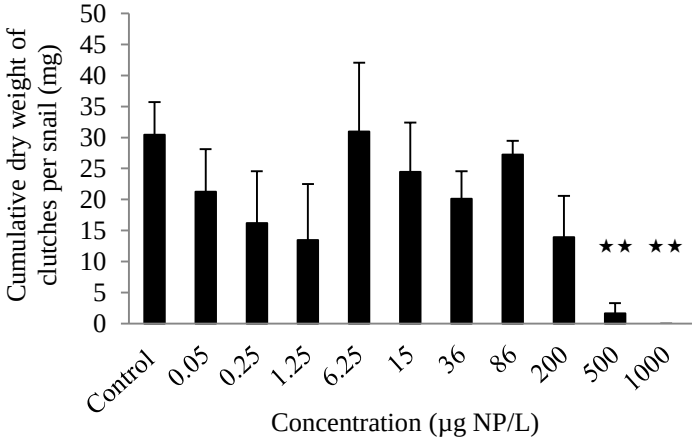


Cumulative number of clutches

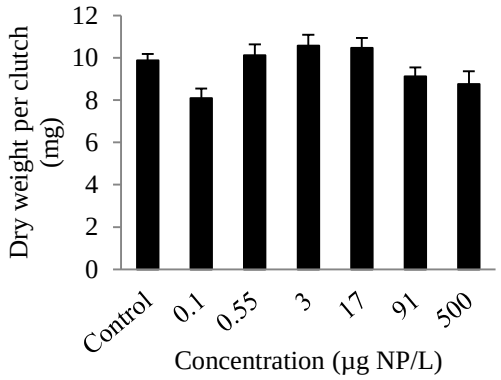




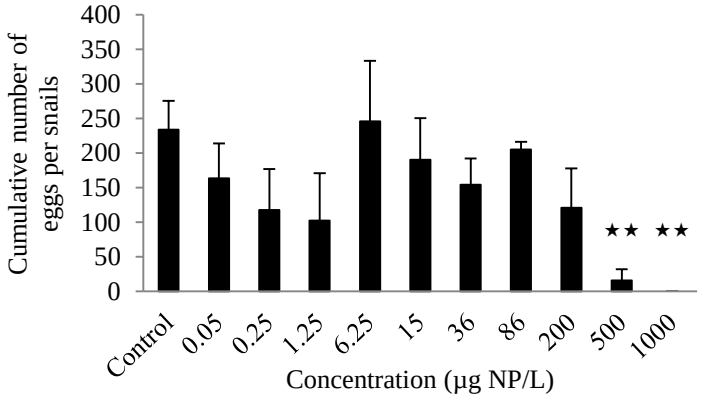
Cumulative dry weight of clutches



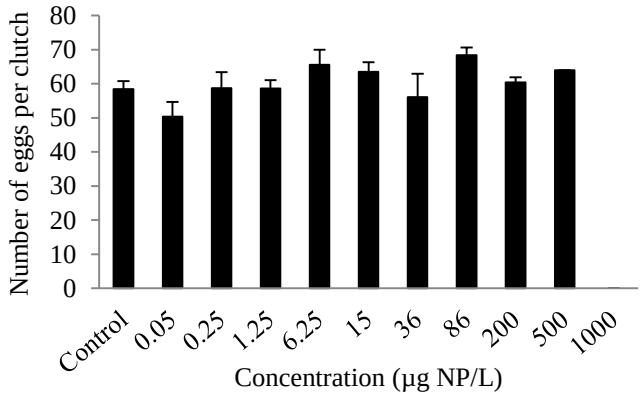
Dry weight per clutch



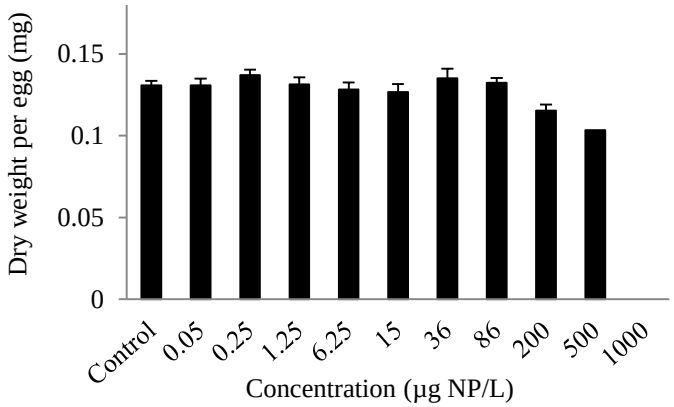
Cumulative number of eggs



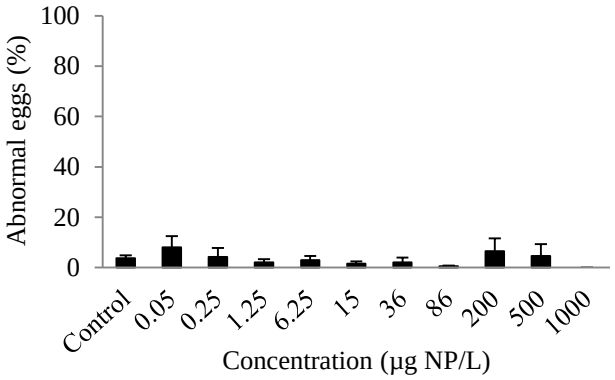
Number of eggs per clutch

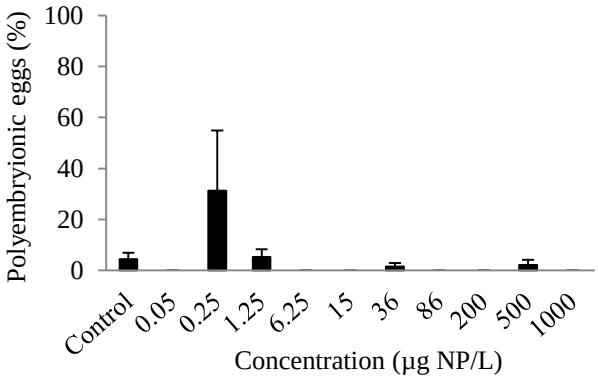


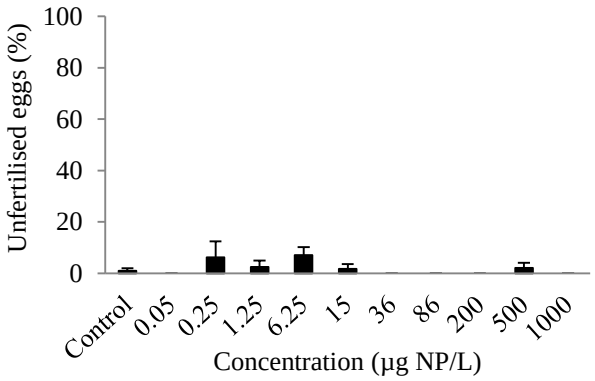
Dry weight per egg

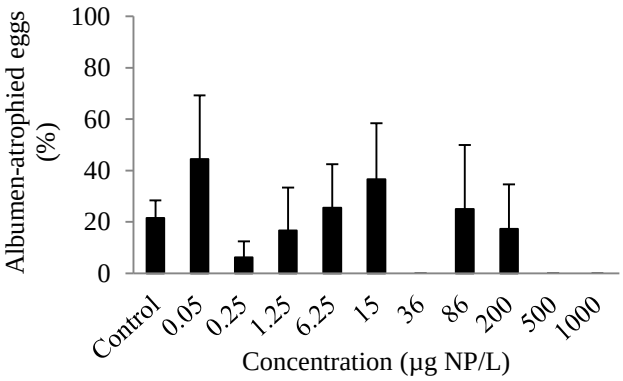


Egg abnormalities

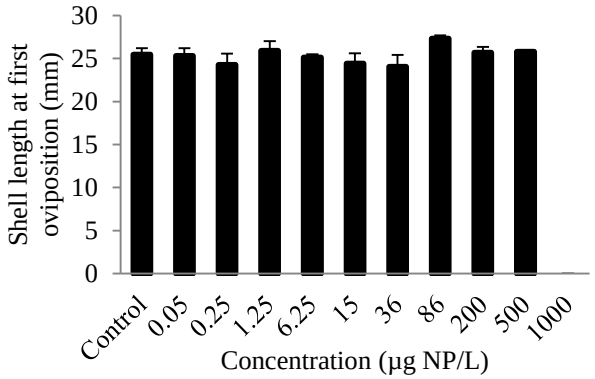


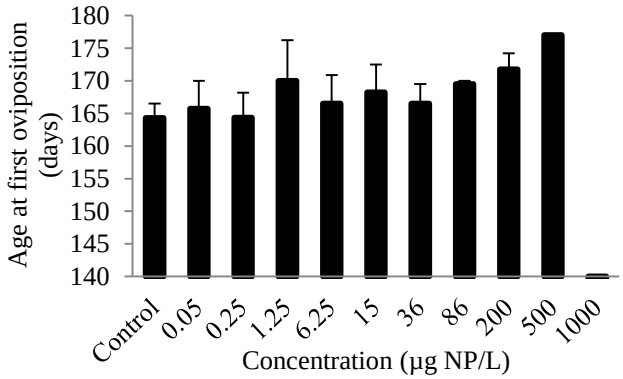






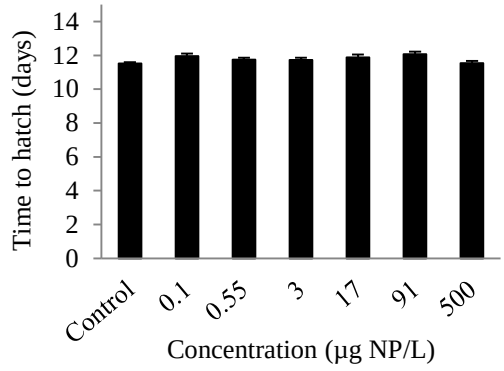
Shell length and age at first oviposition



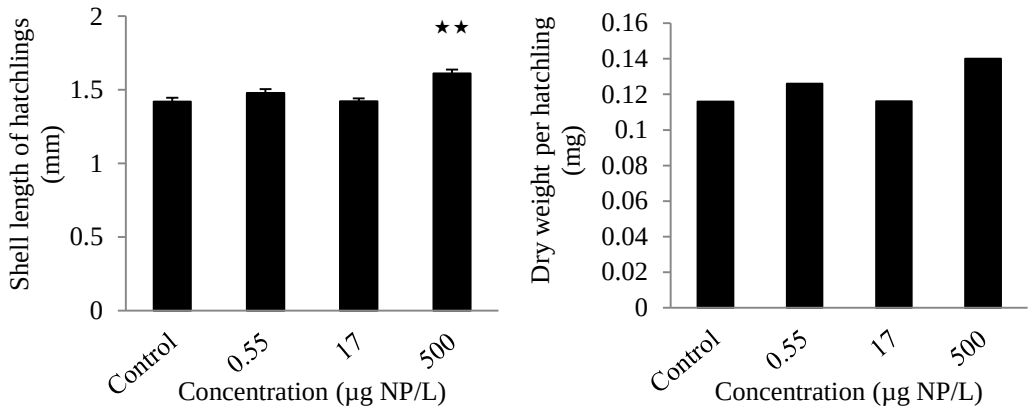


2.2. Non-exposed offspring of treated snails

Time to hatch (in clean water)



Shell length and dry weight of hatchlings. Clutches were collected on 54th day of the test.



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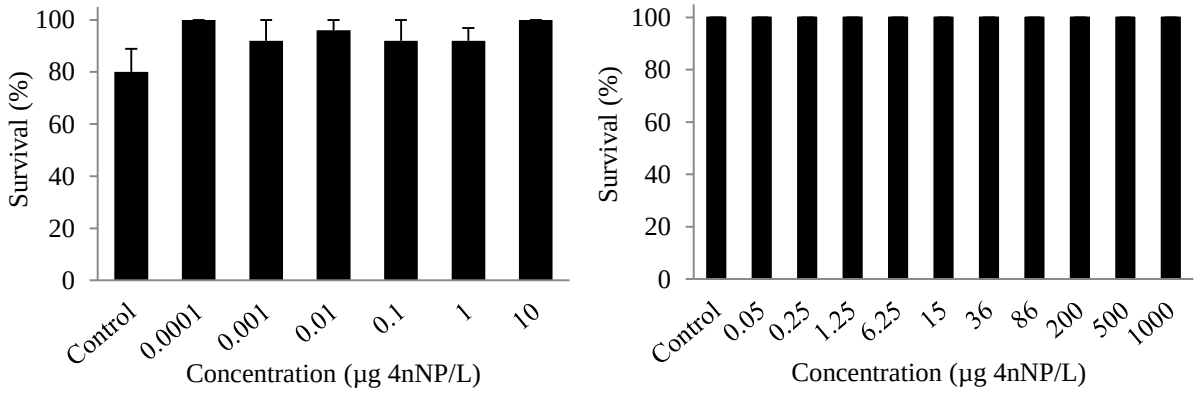
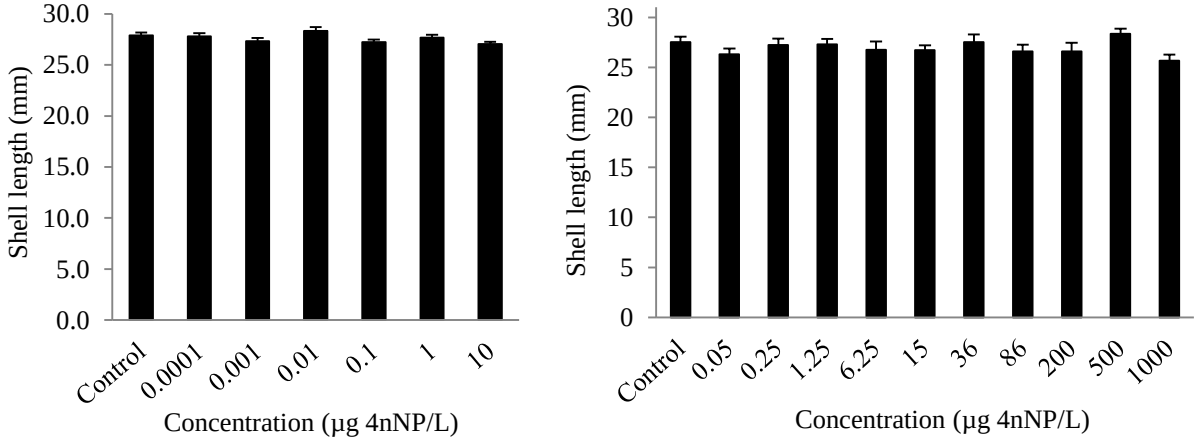
3. 4-n-Nonylphenol (4nNP)

3.1 Adults and sub-adults

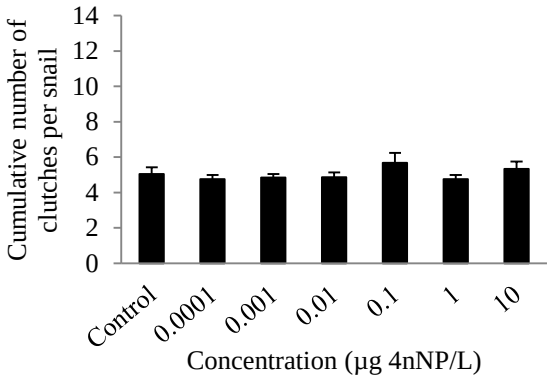
Survival

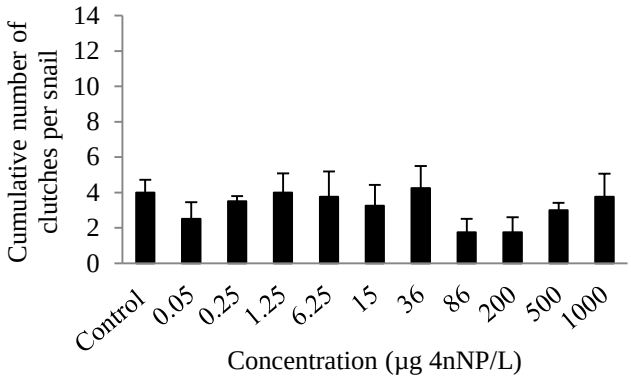


Shell length

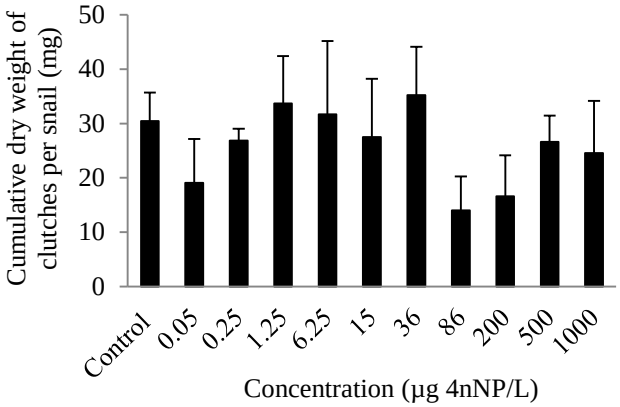


Cumulative number of clutches

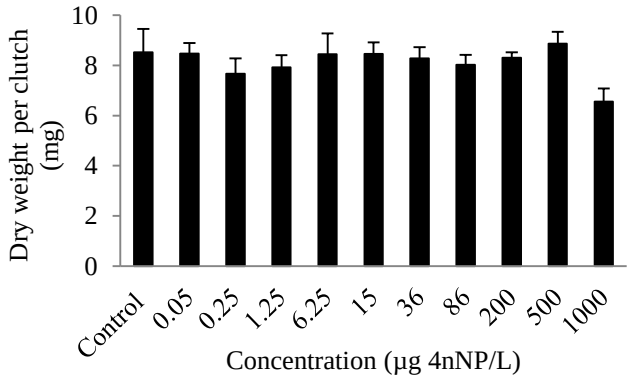




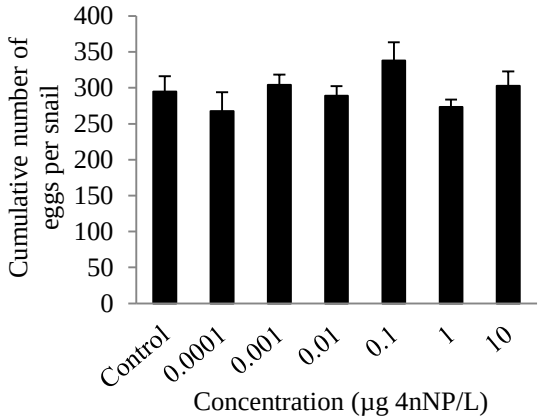
Cumulative dry weight of clutches

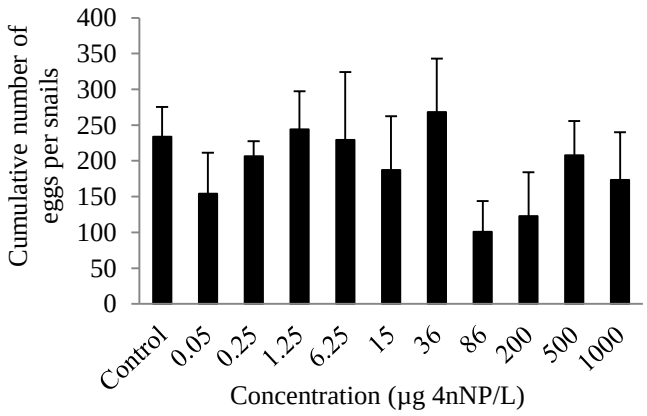


Dry weight per clutch

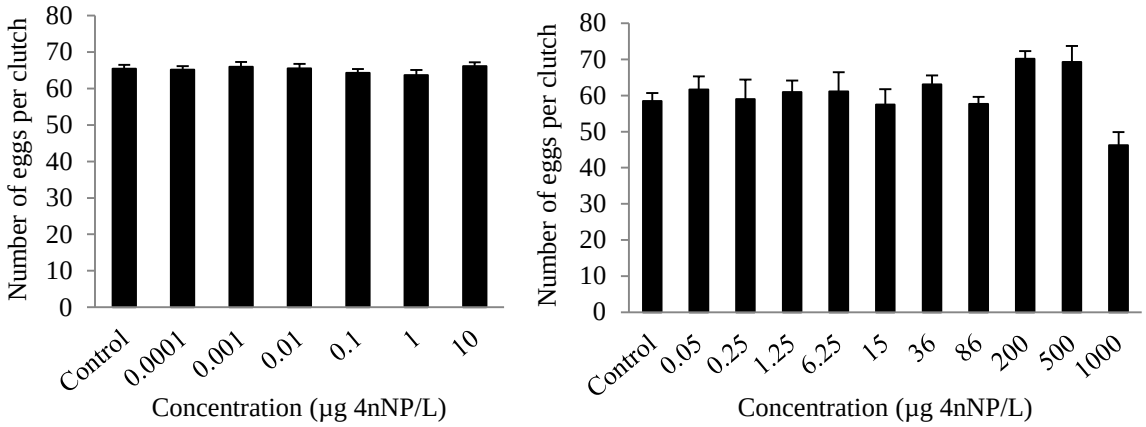


Cumulative number of eggs

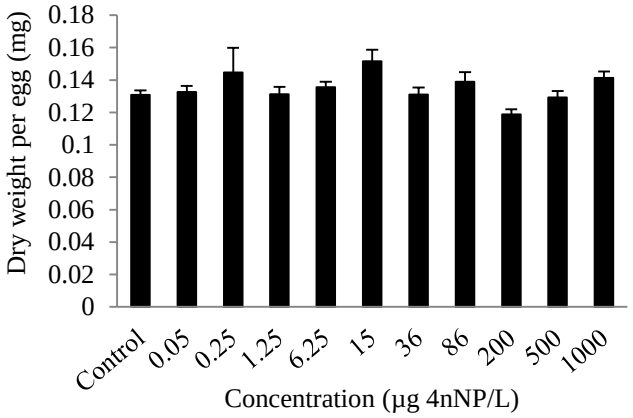




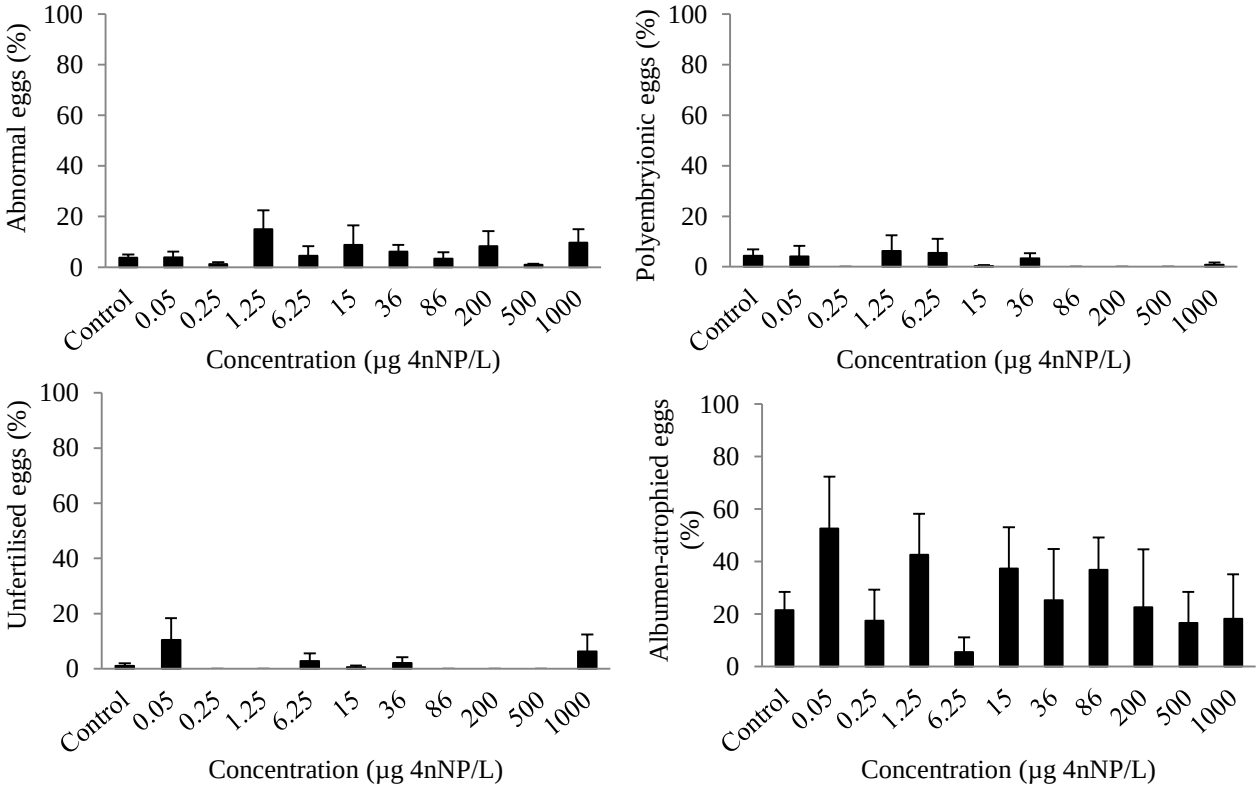
Number of eggs per clutch



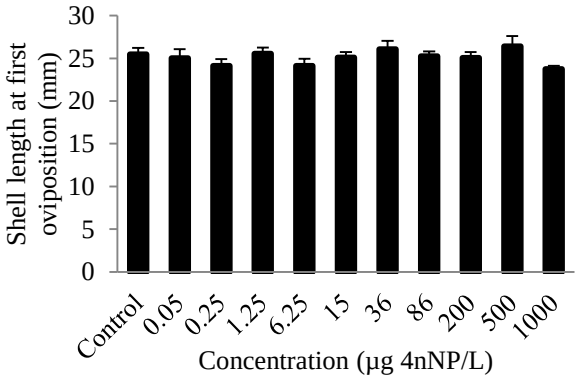
Dry weight per egg

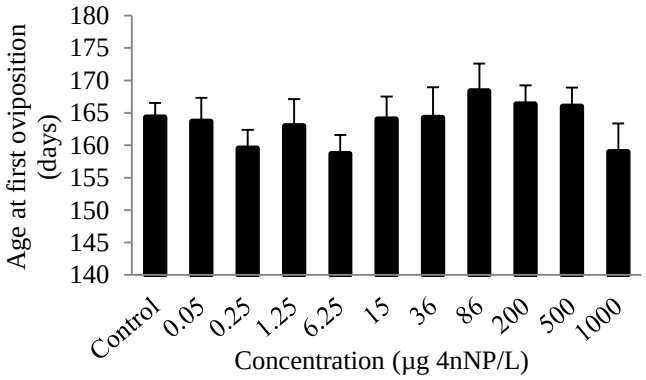


Egg abnormalities



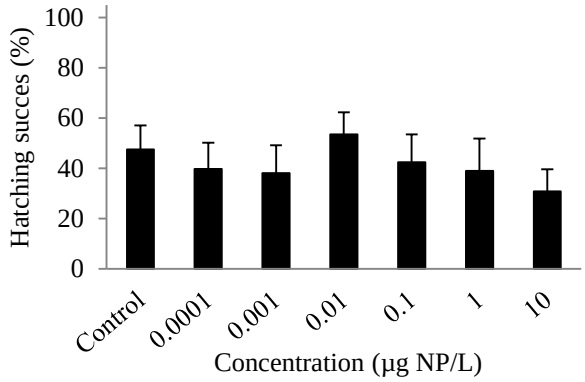
Shell length and age at first oviposition





3.2. Non-exposed offspring of treated snails

Hatching success. Clutches were collected during 4th week of the test. Hatching success was evaluated after a 35 days. During this time clutches stayed in clean water.



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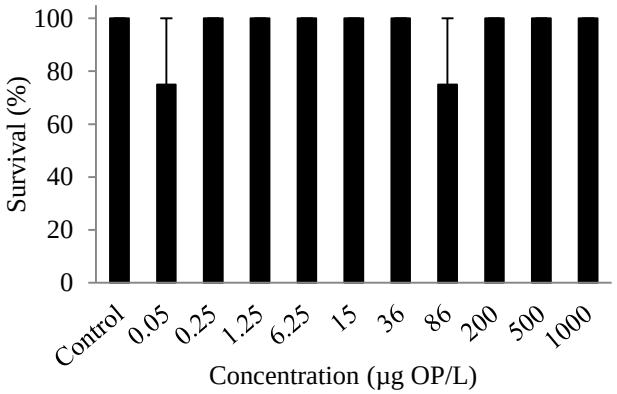
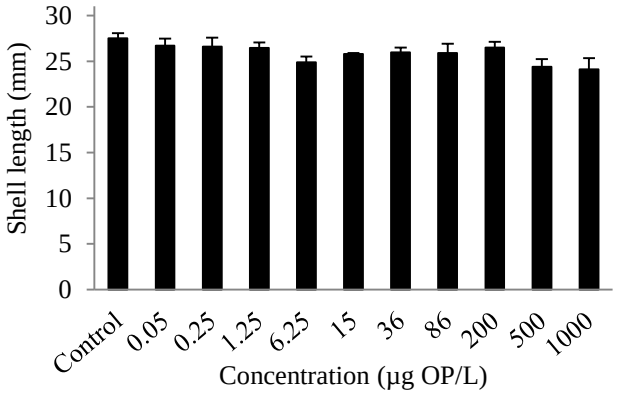
4. 4-tert-octylphenol (OP)

4.1 Adults and sub-adults

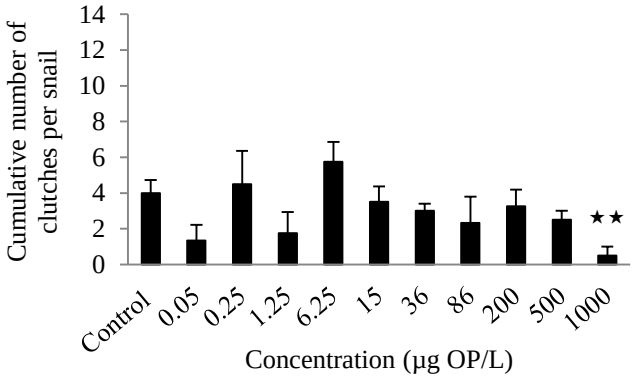
Survival

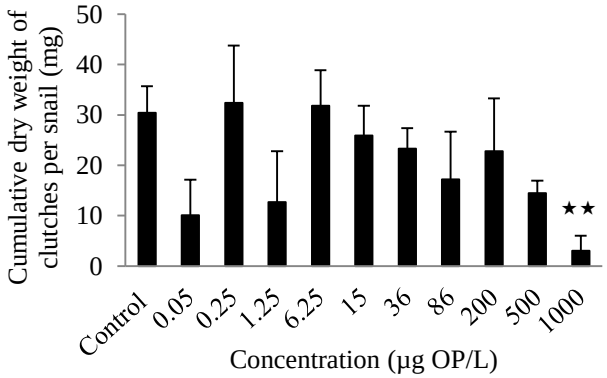


Shell length

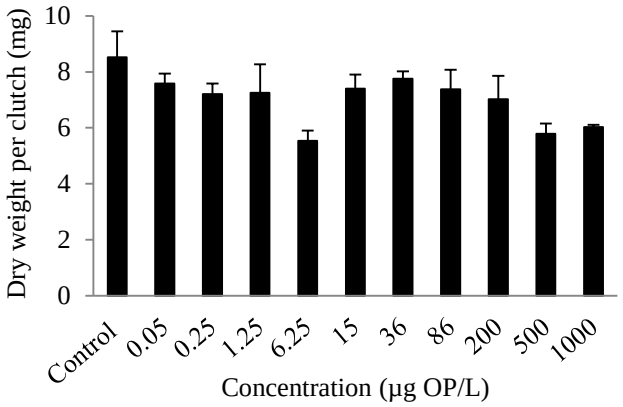


Cumulative number of clutches and cumulative dry weight of clutches

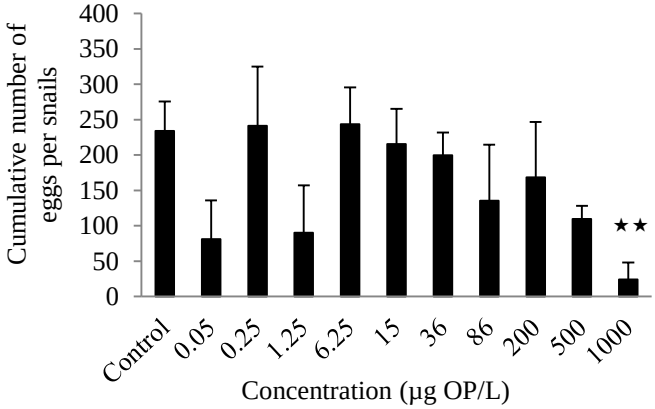




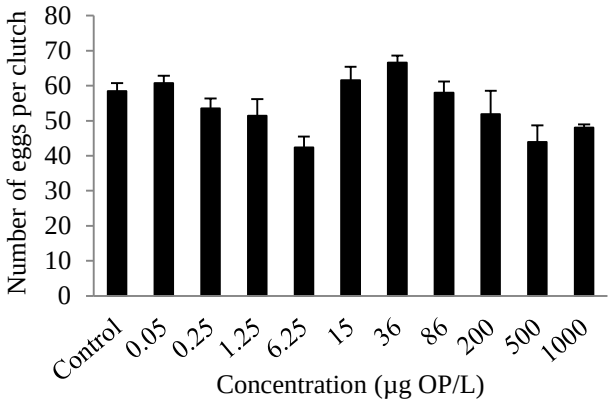
Dry weight per clutch

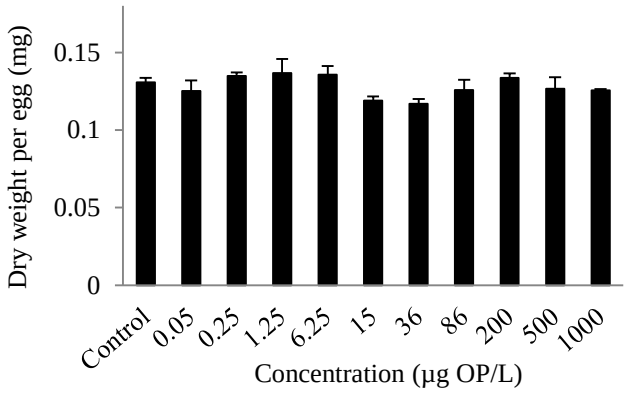


Cumulative number of eggs

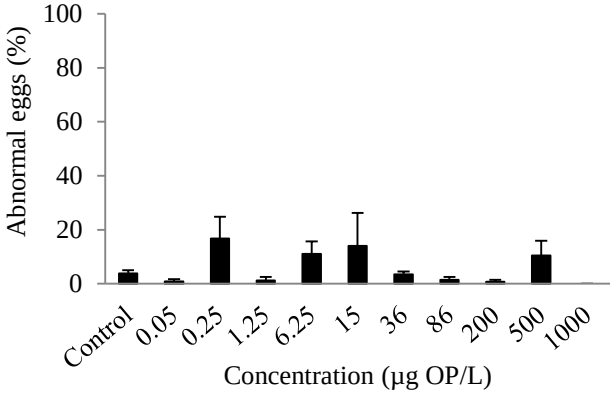


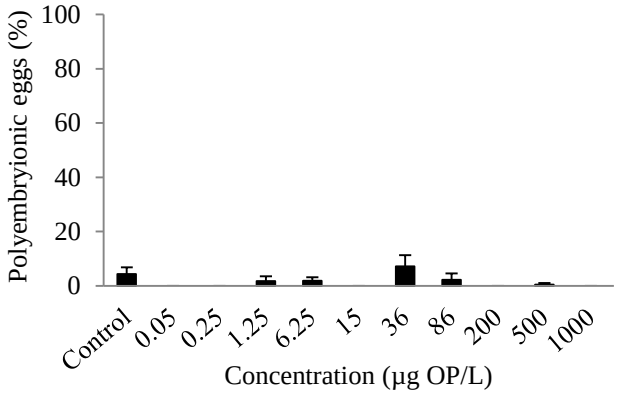
Number of eggs per clutch and dry weight per egg

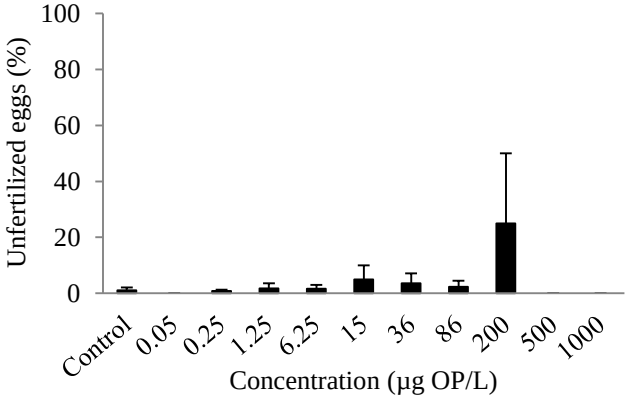


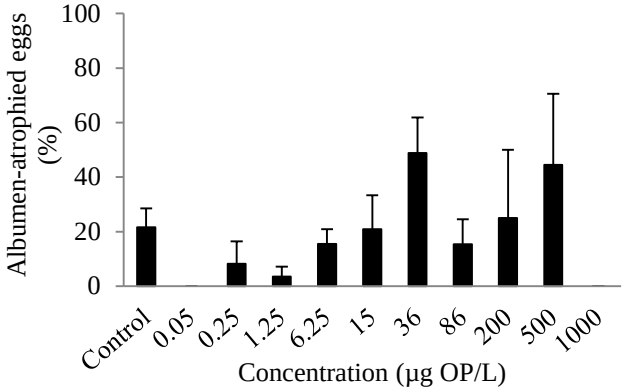


Egg abnormalities

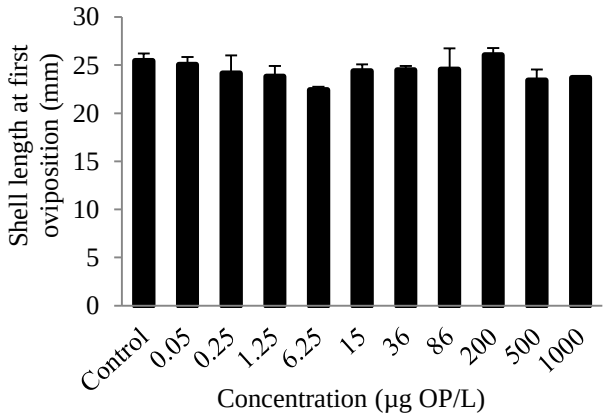


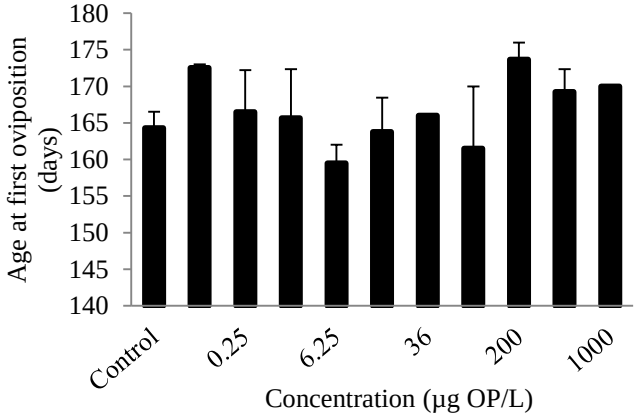






Shell length and age at first oviposition





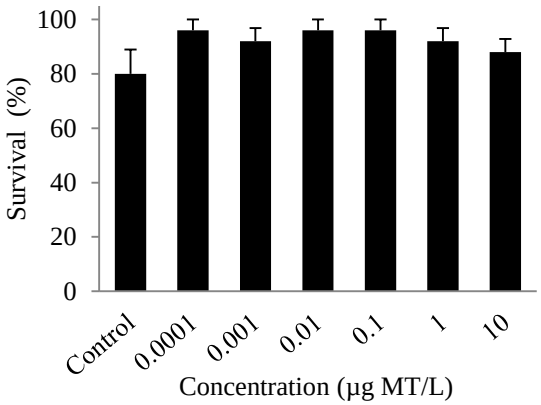
Appendix 5

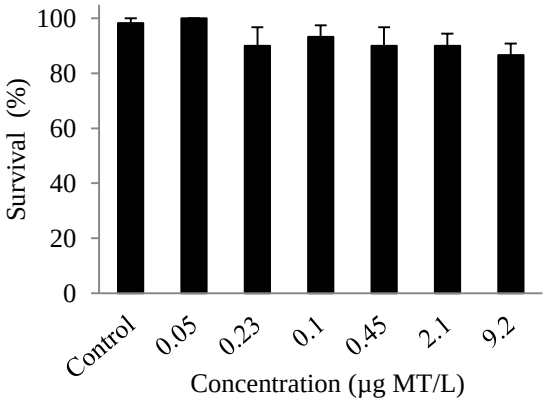
Test results on exposure of Lymnaea stagnalis to 17α-methyltestosterone

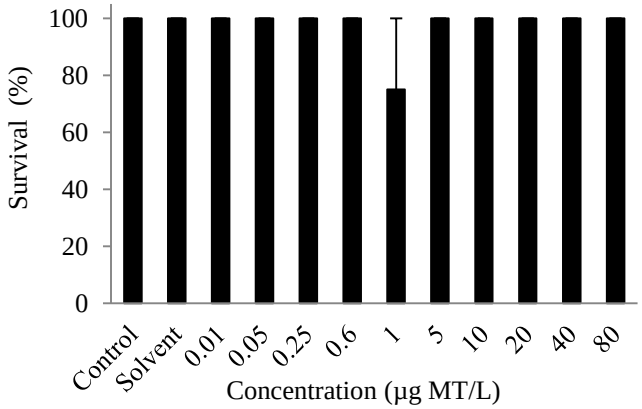
In this appendix, the results from the partial life-cycle toxicity tests in which *L. stagnalis* was exposed to 17α-methyltestosterone (MT) are presented. I performed these toxicity tests during my PhD project along with other tests, such as the EE2 exposure tests (Chapter 5). Therefore, the design of the tests with MT and the statistical analyses are the same as described for EE2 in Chapter 5 and Appendices 3 and 4.

1. Adults and sub-adults

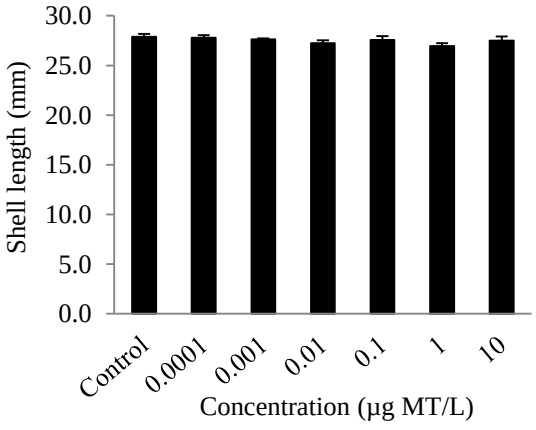
Survival

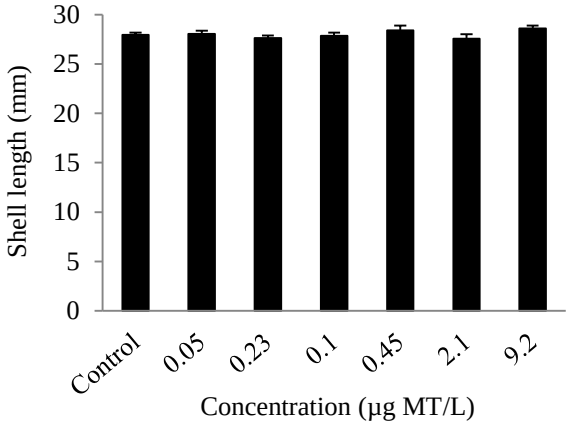


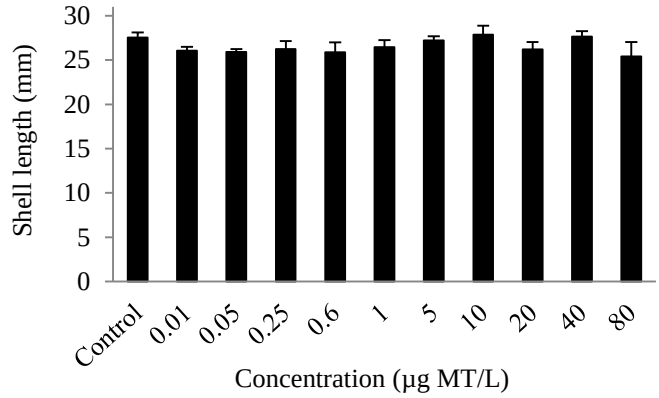




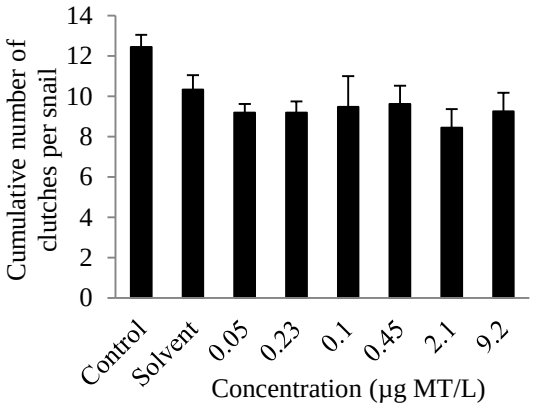
Shell length

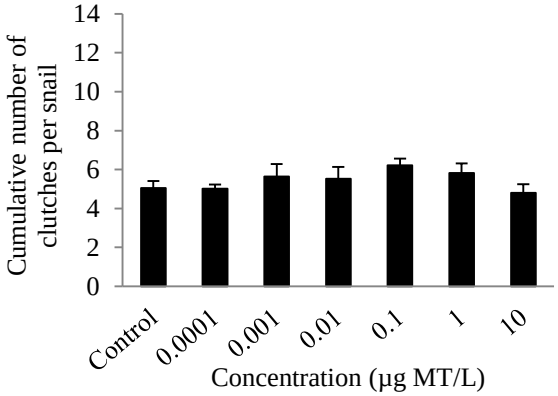


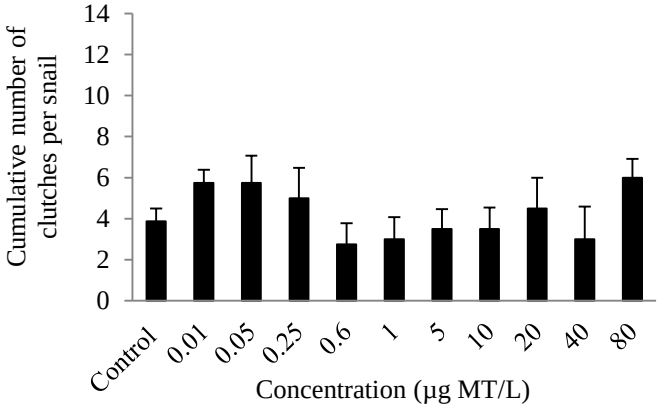




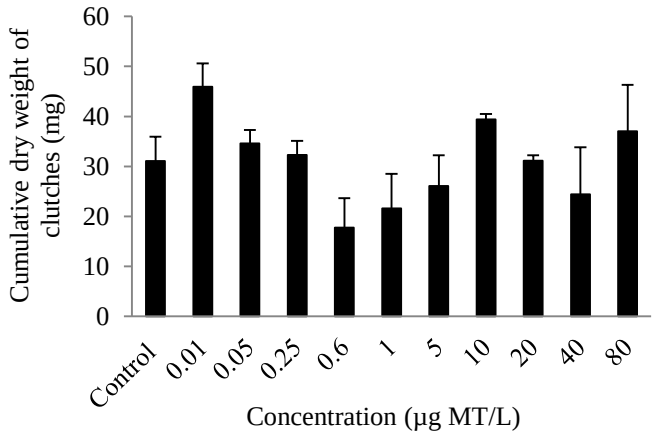
Cumulative number of clutches



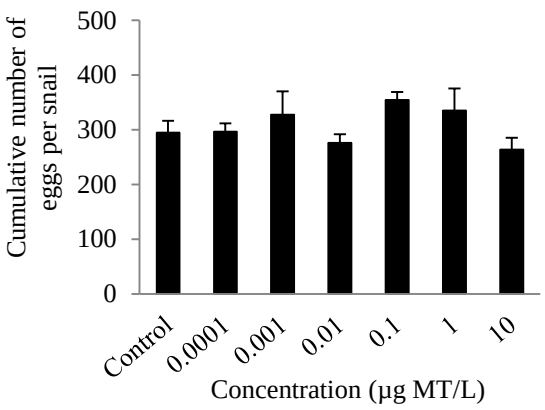


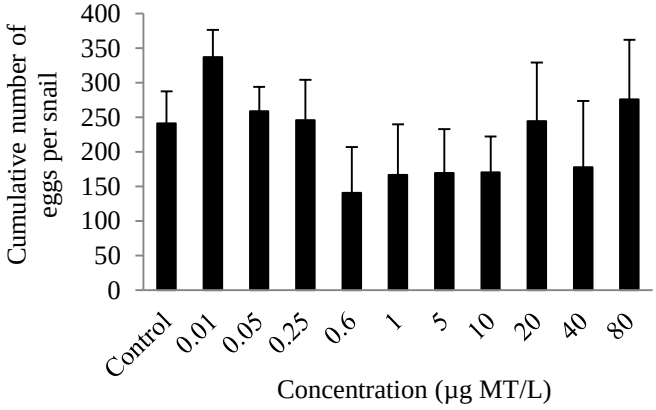


Cumulative dry weight of clutches

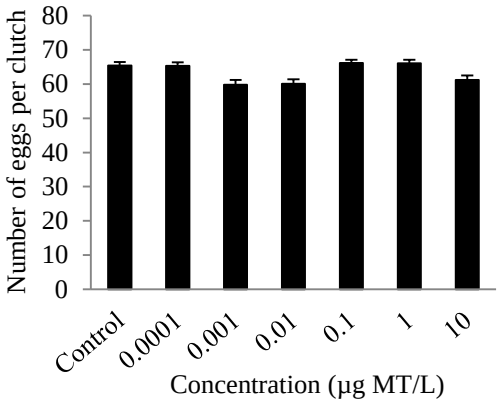


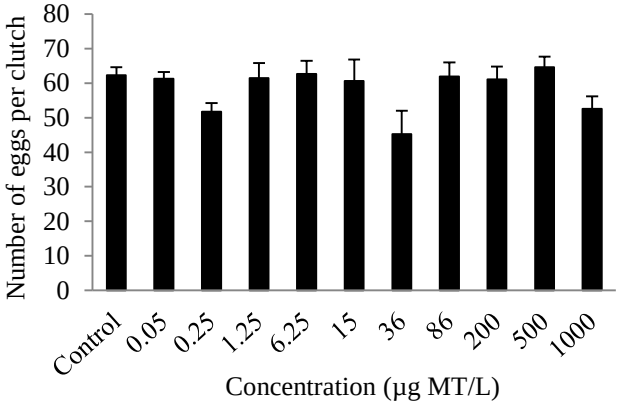
Cumulative number of eggs



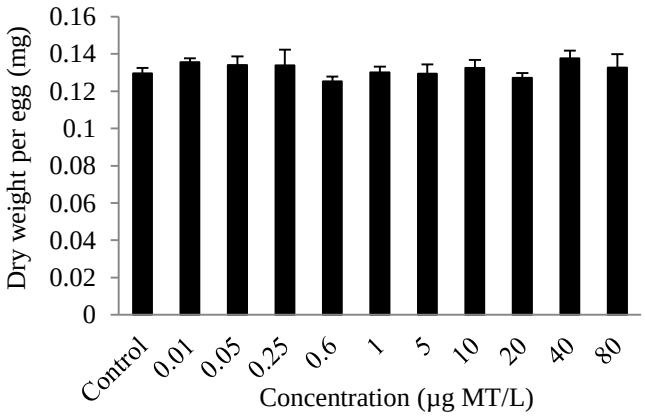


Number of eggs per clutch

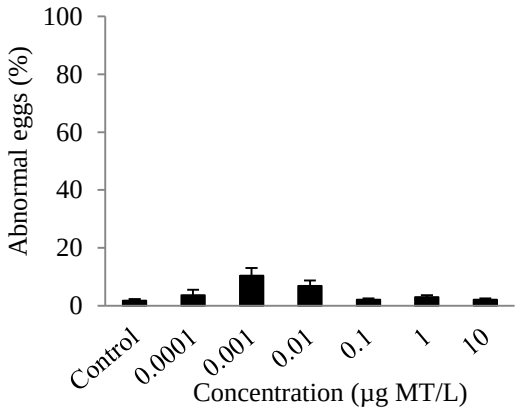


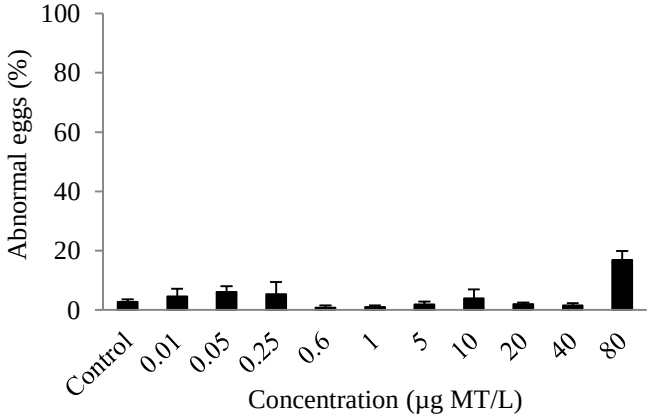


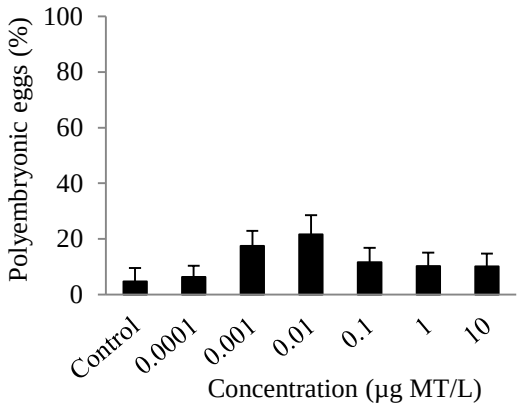
Dry weight per egg

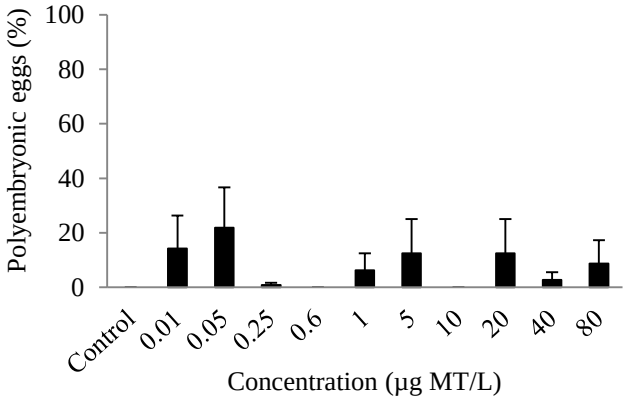


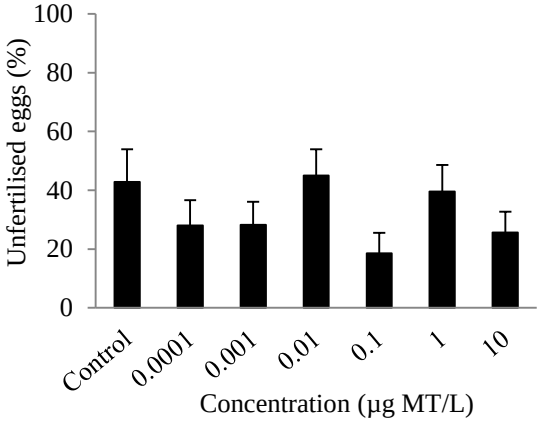
Egg abnormalities

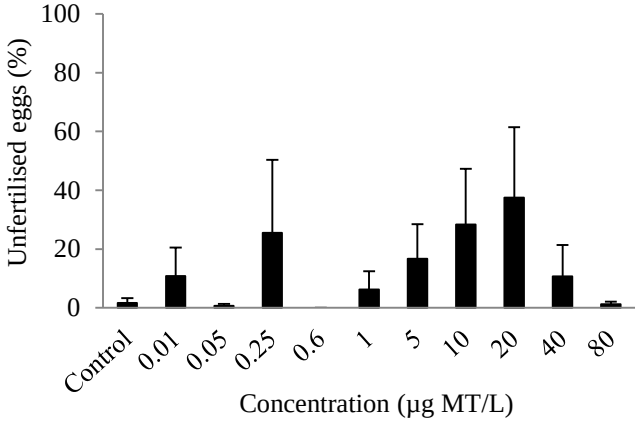


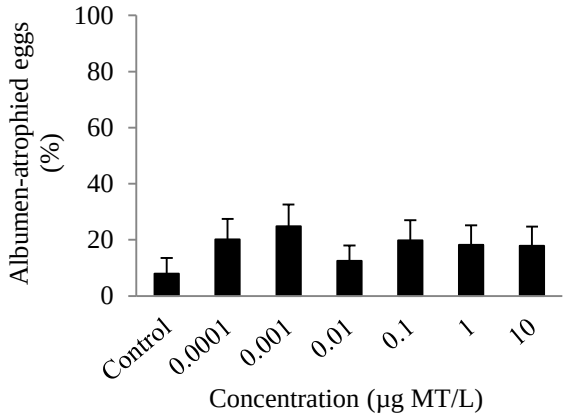


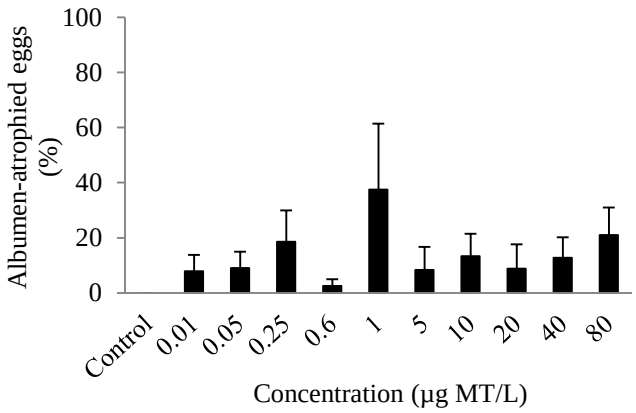




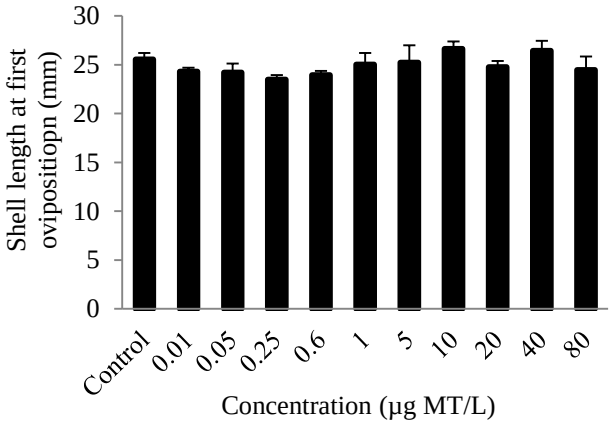


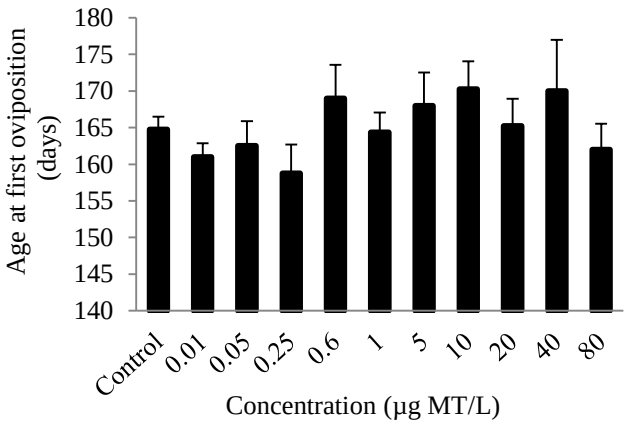






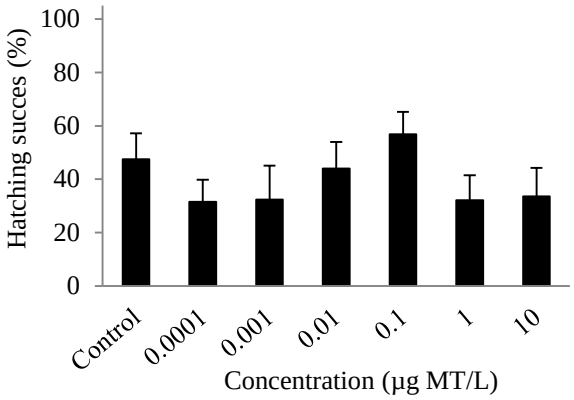
Shell length and age at first oviposition



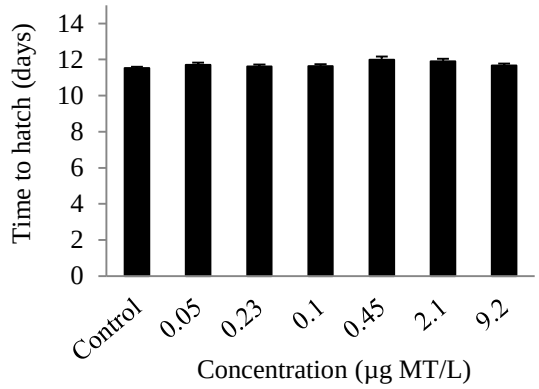


2. Non-exposed offspring of treated snails

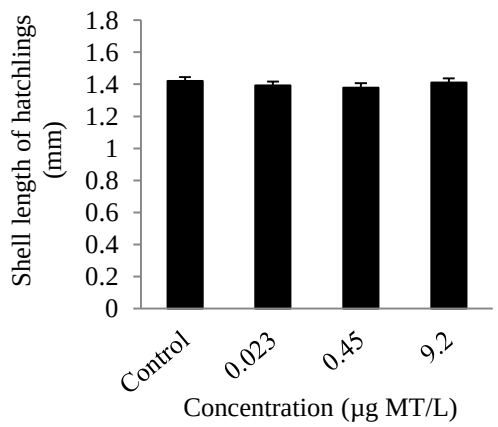
Hatching success. Clutches were collected during 4th week of the test. Hatching success was evaluated after a 35 days. During this time clutches stayed in clean water.

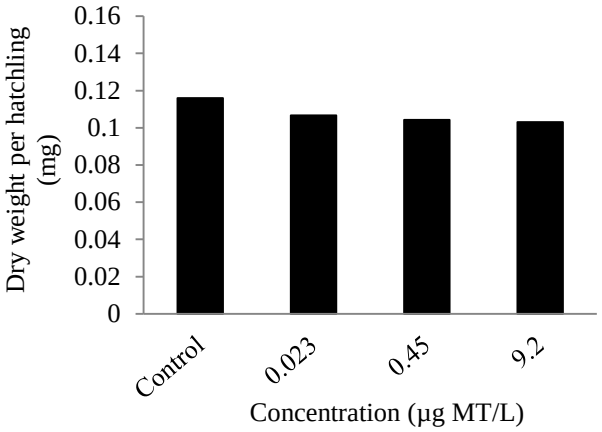


Time to hatch (in clean water)



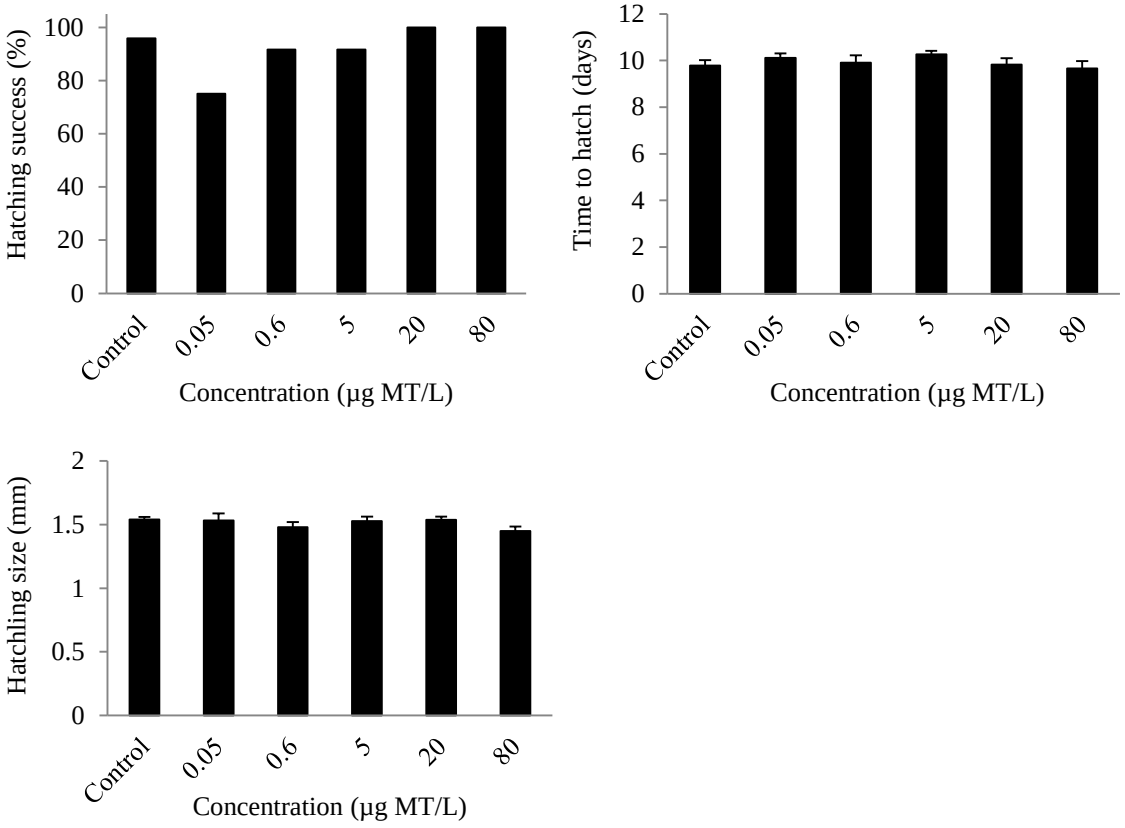
Shell length and dry weight of hatchlings. Clutches were collected on 54th day of the test.





3. Exposed offspring of non-exposed snails.

Hatching success, time to hatch, and hatchling size. Egg clutches were collected from non-exposed snails in laboratory culture. Then eggs were taken out from jelly-masses, each egg was placed into a plastic well, and directly exposed to MT in isolation during 21 days.



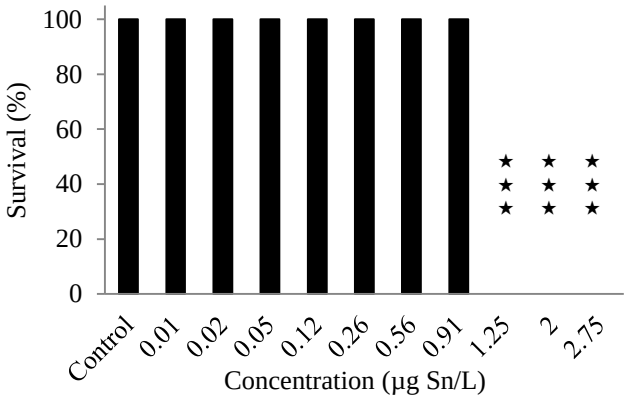
Appendix 6

Test results on exposure of Lymnaea stagnalis to triphenyltin

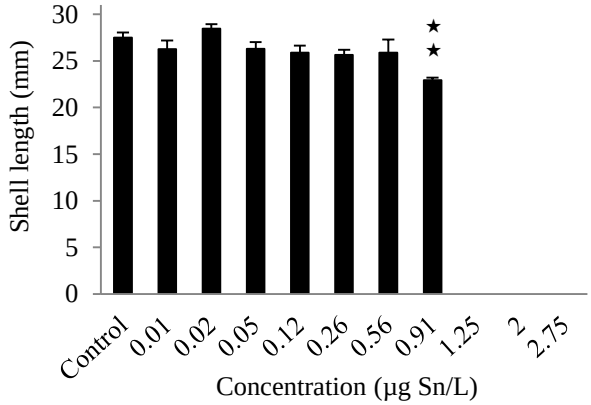
In this appendix, the results from the partial life-cycle toxicity tests in which sub-adults of *L. stagnalis* were exposed to triphenyltin (TPT) during 35 days are presented. This toxicity test was performed along with other tests presented in my thesis, such as the EE2 exposure test with sub-adults and embryos (Chapter 5). Therefore, the design of the TPT exposure test and statistical analyses are the same as described in Chapter 5 and Appendix 3 for exposure tests with sub-adults and embryos.

1. Sub-adults

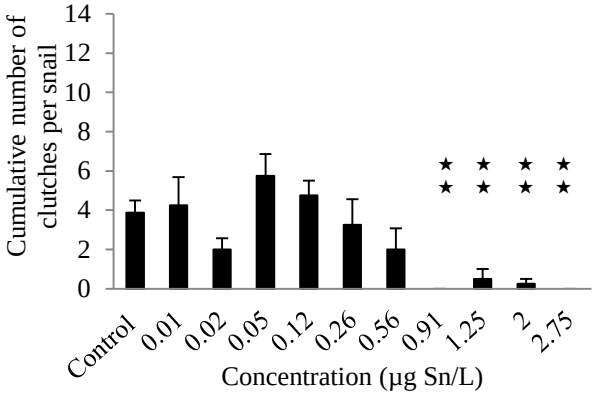
Survival. The average number of days that animals survived exposure to concentrations of 1.25, 2, and 2.75 µg Sn/L were 19, 15, and 11 (SD ± 3.4, 1.3, 3.1), respectively.

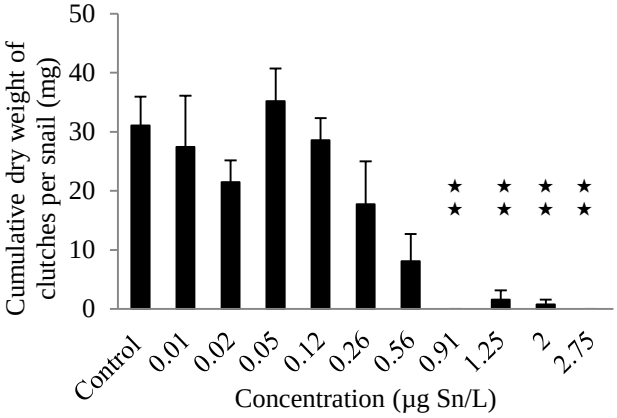


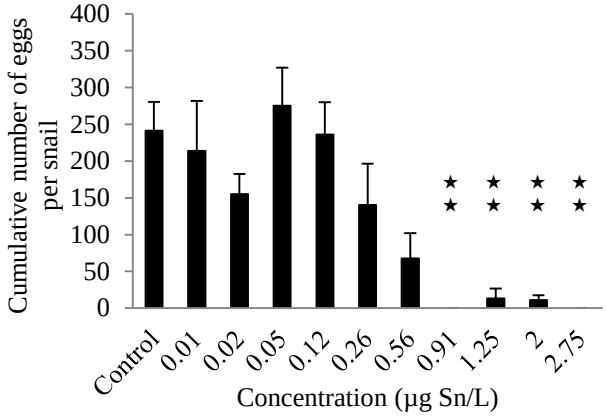
Shell length

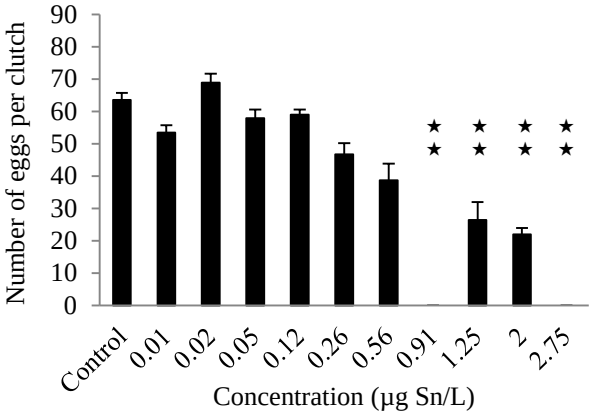


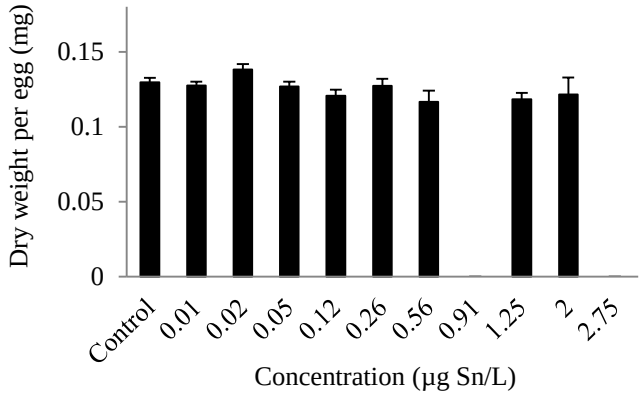
Reproduction



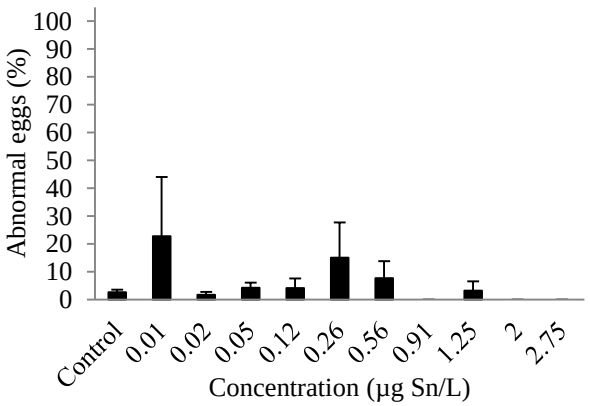


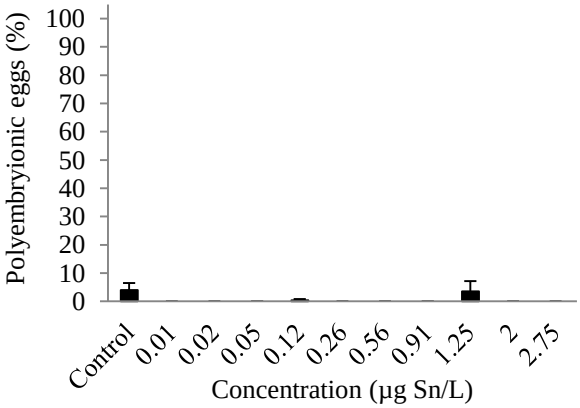


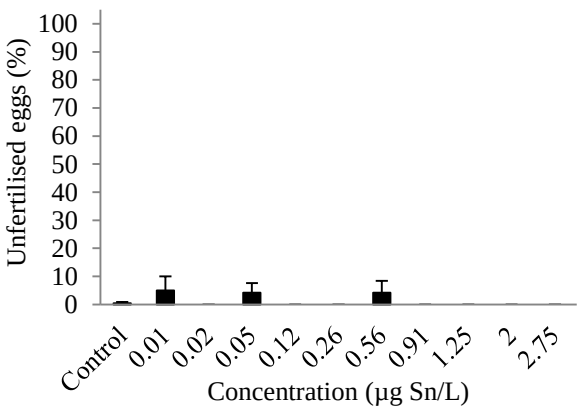


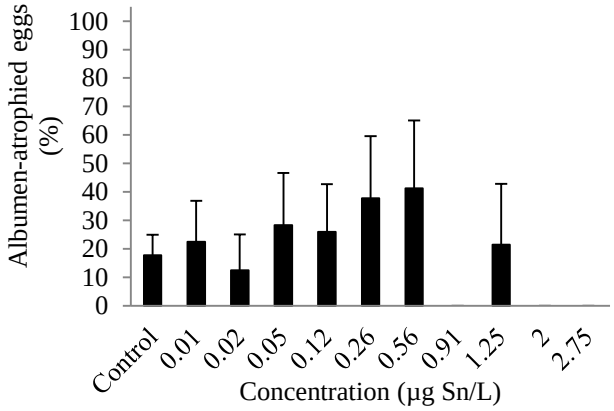


Egg abnormalities

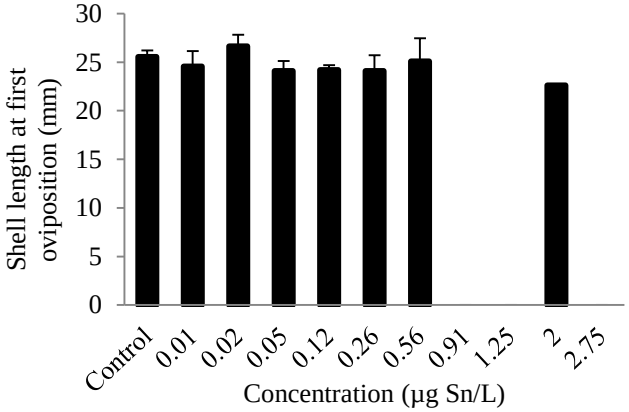


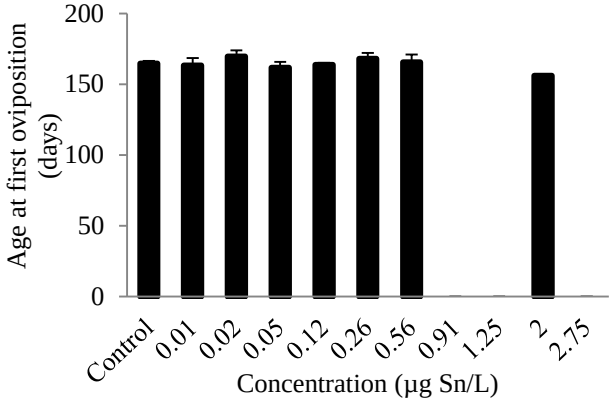






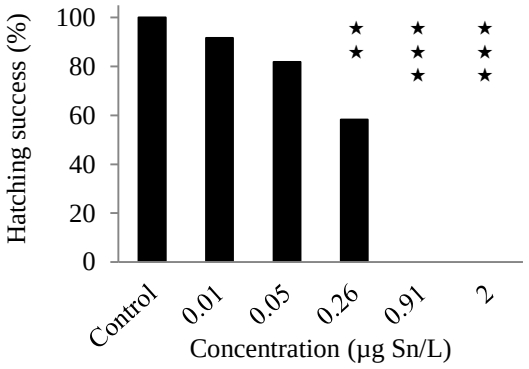
Shell length and age at first oviposition

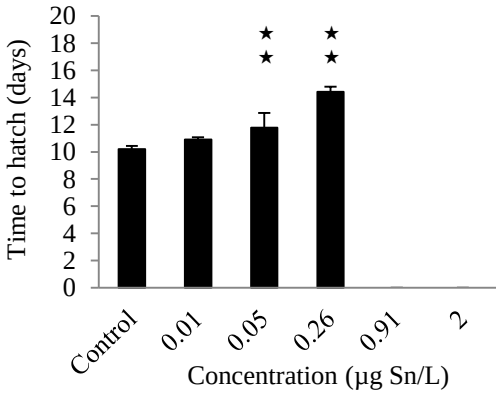


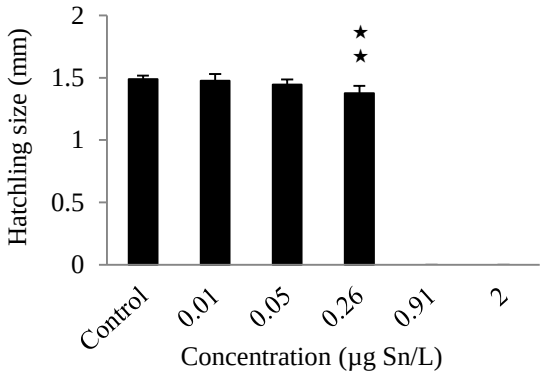


2. Exposed offspring of non-exposed snails.

Hatching success, time to hatch, and hatchling size. Egg clutches were collected from non-exposed snails in laboratory culture. Then eggs were liberated from jelly-masses, each egg was placed into a plastic well, and directly exposed to TPT in isolation during 21 days.







Appendix 7

Effects of solvents commonly used in ecotoxicity testing on Lymnaea stagnalis

Introduction

Many chemicals are poorly soluble in water. To make such chemicals available to aquatic test organisms and allow proper assessments of their toxicity, these chemicals have to be dissolved in the test medium. The OECD [1] recommends methods for dissolution of poorly soluble chemicals in aquatic toxicity testing, which have been adopted for the OECD standardisation of reproduction tests with molluscs [2]. Physical methods for the dissolution should be used first (*e.g.*, stirring, blending, ultrasonication). If the physical methods are not efficient, then solvents can be used in concentration up to 0.1 mL/L (0.01 % v.v.). Other authors suggested 0.02 ml/L as a protective concentration (*e.g.*, [3]). However, some evidence suggests that even lower concentrations may affect some performance of animals in toxicity tests (for a detailed discussion see the review of Lecomte et al. [4]).

The purpose of our test was to examine effects of solvents that are commonly used as carriers for chemicals in ecotoxicity tests. Particular attention was paid on whether solvent are toxic at the recommended safety levels. The findings from our tests provide complementary information about effects of carrier solvents on *L. stagnalis* that may be useful for the OECD standardisation.

Materials and methods

Principle of the test. Groups of five snails in three replicates (15 snails in total) were tested in each treatment. Snails were exposed to five concentrations of solvents during 35 days. Survival, growth, and reproduction were monitored. The test was conducted under semi-static conditions, with water renewal twice a week. Snails were tested in glass beakers filled with 500 mL of test water, under a photo period of 14:10 hours light:dark and temperature of 21° C, with organic lettuce as a food source provided *ad libitum*. Acclimation period was 7 days.

Test animals. The test was conducted with juvenile snails of *L. stagnalis* of initial mean shell length 19.9 ± 2.2 mm (SD). At this size snails still do not lay eggs, which was confirmed by our observations from the lab culture as well as during the acclimation period. During the test most of snails became mature.

Toxicants. Snails were exposed to the following solvents: acetone, acetonitrilie, dimethyl sulfoxide, ethanol, glacial acetic acid, tert-butanol, and triethylene glycol. Tested concentrations were 0.02, 0.1, 0.5, 2.5, and 12.5 mL/L.

Endpoints:

1. **Survival** (%) was monitored daily.
2. **Shell length** (mm) was used as a proxy for growth. Individual values were obtained at days 0 and 35 of the experiment. Shell lengths were expressed as the mean value per replicate.
3. **Cumulated number of clutches per snail** (oviposition).
4. **Cumulated number of eggs per snail** (fecundity).

For the last two endpoints clutches were collected twice a week during water renewals, and eggs were counted under the stereo microscope.

Statistical analysis. Test replicates were treated as experimental units. The exact Jonckheere-Terpstra trend test was applied for testing all endpoints at a significance level of $\alpha = 0.05$, except initial shell length of snails. Mean shell sizes in acetonitrile, dimethyl sulfoxide, and glacial acetic acid treatments were significantly smaller comparing with the control data ($p = 0.039$, $p = 0.029$, and $p = 0.037$, respectively, $\alpha = 0.05$). To accommodate this difference, growth was expressed as a percentage of the increase of the mean shell length in each replicate during 35 days (Table 4). Initial

shell lengths of snails in treatments were tested for differences relative to shells of control snails using the ANOVA test. Results of the statistical analysis were used to define no observed effect concentrations (NOECs) of solvents for each endpoint. Analyses were performed in SPSS (v. 20) software (IBM SPSS, Armonk, NY).

Results

Results on NOECs are summarised in Table 1. Averaged data obtained from the toxicity tests are summarised in Tables 3-7.

Table 1. No observed effect concentrations (NOECs, mL/L) of different solvents (columns) on tested endpoints (rows).

Chemical	Acetone	Acetonitrile	Dimethyl sulfoxide	Ethanol	Glacial acetic acid	Tert-butanol	Triethylene glycol
Survival (%)	> 12.5	2.5	> 12.5	2.5	0.1	2.5	> 12.5
Shell length increase (%)	2.5	2.5	> 12.5	0.5	> 0.1	0.1	> 12.5
Oviposition	0.5	2.5	0.5	0.02	> 0.1	0.02	> 12.5
Fecundity	0.5	2.5	0.5	0.02	> 0.1	0.02	> 12.5

Table 2. Percentage of surviving animals by the end of the test.

Replicate		Acetone (mL/L)					Acetonitrile (mL/L)					Dimethyl sulfoxide (mL/L)				
		0.02	**0.1**	**0.5**	**2.5**	**12.5**	**0.02**	**0.1**	**0.5**	**2.5**	**12.5**	**0.02**	**0.1**	**0.5**	**2.5**	**12.5**
Day 0-35	**A**	100	100	100	100	100	100	100	80	100	20	100	100	100	100	100
	B	100	100	100	100	100	100	100	100	100	0	100	100	100	100	100
	C	100	100	100	100	100	100	80	100	100	20	100	100	100	100	100
Replicate		**Ethanol (mL/L)**					**Glacial acetic acid (mL/L)** *					**Tert-butanol (mL/L)** **				
		0.02	**0.1**	**0.5**	**2.5**	**12.5**	**0.02**	**0.1**	**0.5**	**2.5**	**12.5**	**0.02**	**0.1**	**0.5**	**2.5**	**12.5**
Day 0-35	**A**	100	100	100	80	100	100	100	0	0	0	100	100	100	100	0
	B	100	100	100	100	80	100	100	0	0	0	80	100	80	100	0
	C	100	100	100	100	80	100	100	0	0	0	100	100	100	100	0
Replicate		**Triethylene glycol (mL/L)**					**Control**									
		0.02	**0.1**	**0.5**	**2.5**	**12.5**										
Day 0-35	**A**	100	100	100	100	100	100									
	B	100	100	100	100	100	100									
	C	100	100	100	100	100	100									

* All snails exposed to solvent concentration of 0.5-12.5 mL/L died within two hours after exposure; ** all snails exposed to solvent concentration of 12.5 mL/L died within 7 days.

Table 3. Mean shell length of snails at days 0 and 35. N.A. = Not assessed due to mortality.

Replicate		Acetone (mL/L)					Acetonitrile (mL/L)					Dimethyl sulfoxide (mL/L)				
		0.02	**0.1**	**0.5**	**2.5**	**12.5**	**0.02**	**0.1**	**0.5**	**2.5**	**12.5**	**0.02**	**0.1**	**0.5**	**2.5**	**12.5**
Day 0	**A**	20.3	20.9	20.6	19.6	20.8	19.7	19.9	20.7	19.7	18.5	19.6	19.7	19.2	18.5	19.3
	B	22.1	21.4	21.0	20.1	20.9	19.9	20	18.8	18.8	19.5	20.5	19.1	20.1	19.1	19
	C	19.3	21.1	20.0	21.5	20.8	19.2	19.4	19.4	20.3	18.7	19.1	20.8	20.1	19.4	18.3
Day 35	**A**	25.9	25.6	25.7	27.3	21.3	24.3	25.5	26.8	24.7	17.9	25	24.9	25.2	25.3	22.8
	B	27.3	26.3	26.3	25.9	21.1	25.3	25.2	24.4	24.7	23.4	26	24.3	25.7	25.3	24.1
	C	24.5	26.2	24.8	24.9	21.4	25.1	23.7	26	25.6	20.5	25.1	25.9	26	24.7	23.8
Replicate		**Ethanol (mL/L)**					**Glacial acetic acid (mL/L)**					**Tert-butanol (mL/L)**				
		0.02	**0.1**	**0.5**	**2.5**	**12.5**	**0.02**	**0.1**	**0.5**	**2.5**	**12.5**	**0.02**	**0.1**	**0.5**	**2.5**	**12.5**
Day 0	**A**	19.1	20.5	19	19.7	20.5	19.5	18.9	17.2	19.2	19.2	19.2	20.5	20.2	20.5	18.6
	B	19.3	20.8	20.3	19.7	19.7	20.9	19.8	19.7	20.5	18.7	20.4	20.8	20.4	21.2	18.9
	C	20.7	20.5	18.8	19.2	20.3	19	20	20.3	19.2	20.2	20.9	20.7	20.1	19.4	20.1
Day 35	**A**	25.1	26.4	24.9	23.3	20.8	25.1	23.6	N.A.	N.A.	N.A.	25.3	25.7	24.3	24.5	N.A.
	B	24.8	25.1	25.3	23.5	19.7	25.3	24.2	N.A.	N.A.	N.A.	25.2	26	25	24.8	N.A.
	C	26.3	25.1	25.8	22	20.5	25.4	24.2	N.A.	N.A.	N.A.	25.8	26.3	25.5	24.7	N.A.

Replicate		Triethylene glycol (mL/L)					Control
		0.02	**0.1**	**0.5**	**2.5**	**12.5**	
Day 0	**A**	20.4	20.4	19.9	20	21.3	20.6
	B	20.6	20.5	19.6	19.7	20.9	20.8
	C	19.9	20.9	18.8	18.8	20.7	20.7
Day 35	**A**	25.9	24.8	26.7	25.1	25.5	26.6
	B	25.7	25.1	25.9	25.7	25.3	26.8
	C	25	27.2	26	24.2	25.1	26.8

Table 4. Percentage of the mean shell length increase in 35 days. N.A. = Not assessed due to mortality.

Replicate		Acetone (mL/L)					Acetonitrile (mL/L)					Dimethyl sulfoxide (mL/L)				
		0.02	**0.1**	**0.5**	**2.5**	**12.5**	**0.02**	**0.1**	**0.5**	**2.5**	**12.5**	**0.02**	**0.1**	**0.5**	**2.5**	**12.5**
Day 0-35	**A**	27.6	22.5	24.7	39.2	2.12	23.6	28.3	29.3	25.5	0	27.3	26.6	30.9	36.4	17.9
	B	23.3	22.9	25	28.8	0.9	27.2	26.3	30.3	31.2	20.1	27	27.7	27.7	32.6	27
	C	26.5	24.4	23.8	15.9	2.6	30.5	22.3	33.8	25.7	9.28	31.4	24.6	29.2	27.5	29.9
Replicate		**Ethanol (mL/L)**					**Glacial acetic acid (mL/L)**					**Tert-butanol (mL/L)**				
		0.02	**0.1**	**0.5**	**2.5**	**12.5**	**0.02**	**0.1**	**0.5**	**2.5**	**12.5**	**0.02**	**0.1**	**0.5**	**2.5**	**12.5**
Day 0-35	**A**	31	28.6	31.2	17.9	1.55	28.9	24.9	N.A.	N.A.	N.A.	31.5	25.2	20.5	19.4	N.A.
	B	28.7	20.5	24.7	19.3	0	20.9	22	N.A.	N.A.	N.A.	23.3	25.3	22.7	17	N.A.
	C	26.7	22.2	37.1	14.2	1.07	33.6	21.2	N.A.	N.A.	N.A.	23.8	27.4	27.3	27.2	N.A.

Replicate		Triethylene glycol (mL/L)					Control
		0.02	**0.1**	**0.5**	**2.5**	**12.5**	
Day 0-35	**A**	27.3	22	34.2	25.3	19.6	29.3
	B	25.1	22.5	32.4	30.8	20.6	28.9
	C	25.4	30.2	38.4	28.7	21.5	29.6

Table 5. Cumulated number of clutches per snail during 35 days. N.A. = Not assessed due to mortality.

Replicate		Acetone (mL/L)					Acetonitrile (mL/L)					Dimethyl sulfoxide (mL/L)				
		0.02	**0.1**	**0.5**	**2.5**	**12.5**	**0.02**	**0.1**	**0.5**	**2.5**	**12.5**	**0.02**	**0.1**	**0.5**	**2.5**	**12.5**
Day 0-35	**A**	0.8	0.8	0.2	0	0	0	0.6	1	0.4	0	0.2	0	0	0	0.2
	B	0	0.6	0.4	0	0	0.4	0.6	0	0	0	0	0	0.6	0	0.2
	C	0.2	0.2	0.8	0	0	0.4	0	0.8	0.2	0	0.6	0.4	0.4	0.2	0
Replicate		**Ethanol (mL/L)**					**Glacial acetic acid (mL/L)**					**Tert-butanol (mL/L)**				
		0.02	**0.1**	**0.5**	**2.5**	**12.5**	**0.02**	**0.1**	**0.5**	**2.5**	**12.5**	**0.02**	**0.1**	**0.5**	**2.5**	**12.5**
Day 0-35	**A**	0.4	0	0.4	0	0	1	0.4	N.A.	N.A.	N.A.	1	0	0.2	0	0
	B	0.4	0	0.4	0	0	0.6	0.2	N.A.	N.A.	N.A.	0.75	0	0.25	0	0
	C	0.4	0.2	0.2	0	0	0	1.2	N.A.	N.A.	N.A.	0.4	0.4	0.4	0	0

Replicate		Triethylene glycol (mL/L)					Control
		0.02	**0.1**	**0.5**	**2.5**	**12.5**	
Day 0-35	**A**	0.4	0	0.6	0.6	0.6	0.8
	B	0	0.6	0.4	0.8	0.2	0.4
	C	0.4	0.2	0.4	0.4	0.6	0.8

Table 6. Cumulated number of eggs per snail during 35 days. N.A. = Not assessed due to mortality.

Replicate		Acetone (mL/L)					Acetonitrile (mL/L)					Dimethyl sulfoxide (mL/L)				
		0.02	**0.1**	**0.5**	**2.5**	**12.5**	**0.02**	**0.1**	**0.5**	**2.5**	**12.5**	**0.02**	**0.1**	**0.5**	**2.5**	**12.5**
Day 0-35	**A**	49	31	0	0	0	0	45	57	16	0	8.4	0	0	0	13
	B	0	31	16	0	0	25	29	0	0	0	0	9.8	34	0	0
	C	6.2	10	39	0	0	18	0	43	12	0	23	20	26	5.8	0
Replicate		**Ethanol (mL/L)**					**Glacial acetic acid (mL/L)**					**Tert-butanol (mL/L)**				
		0.02	**0.1**	**0.5**	**2.5**	**12.5**	**0.02**	**0.1**	**0.5**	**2.5**	**12.5**	**0.02**	**0.1**	**0.5**	**2.5**	**12.5**
Day 0-35	**A**	14	0	19	0	0	49	21	N.A.	N.A.	N.A.	47	0	7.6	0	0
	B	22	0	28	0	0	29	9.6	N.A.	N.A.	N.A.	51	0	14	0	0
	C	25	12	12	0	0	0	44	N.A.	N.A.	N.A.	34	25	31	0	0

Replicate		Triethylene glycol (mL/L)					Control
		0.02	**0.1**	**0.5**	**2.5**	**12.5**	
Day 0-35	**A**	13	0	37	39	37	43
	B	0	34	25	46	12	23
	C	33	8.2	14	21	26	36

Conclusions

Based upon the obtained values of the NOEC for all monitored endpoints (Table 1), the most adverse solvent was tert-butanol, which exerted toxicity on reproduction at the concentration that is considered sufficiently protective against effects, *i.e.* at 0.1 mL/L [1]. Note that the results on reproduction should be interpreted cautiously because of the high variability of the reproductive outputs of sub-adult/adult snails (Tables 5 and 6). Triethylene glycol did not exert any effect on the test endpoints and can be considered the safest solvent among the seven tested. However, this chemical could not dissolve some of the toxicants that I wanted to test, *e.g.* TBT and TPT. For this reason, I used acetone to dissolve all chemicals I tested for my PhD project.

References

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Summary

Endocrine disruptors (EDs) are exogenous chemicals that induce adverse effect in organisms by interacting with their endocrine system. These chemicals are constituents of many daily life products such as plastic bottles, food, detergents, and cosmetics, and also are ingredients of some plant protection products and biocides. By altering the homeostasis of the endocrine system, EDs primarily impact development, growth, and reproduction of the organism. Those are all crucial determinants of population sustainability, which is the protection goal of risk assessments for aquatic invertebrates. Yet, relatively little is known about invertebrate endocrinology, which complicates the identification and assessment of EDs effects at the individual level. Moreover, the lack of acceptance of mechanistic modelling in ecotoxicological research and environmental risk assessment hinders a proper forecast of the effects of EDs on invertebrate populations. Therefore, this PhD thesis aims at the improvement of the test methods and data analysis tools for assessing and predicting the effects of EDs on the great pond snail *Lymnaea stagnalis*. This aquatic gastropod species has been chosen in my thesis because of its sensitivity to a variety of chemicals, including some EDs. It is also a candidate species for the ongoing standardisation of the reproduction tests with molluscs, coordinated by the Organisation for Economic Development and Co-operation (OECD).

In this thesis, the sensitivity of the great pond snail to androgenic and oestrogenic chemicals, which affect the functions related to the development and maintenance of male or female characteristics, respectively, was investigated. Exposure of the great pond snail to steroid hormones might induce androgenic or oestrogenic effects, similarly to those in vertebrates and some other aquatic snail species. Observing biological responses of the great pond snail to steroid hormones might support the hypothesis that steroid hormonal pathways are involved in the endocrine control of the snail's performance. Furthermore, if exposure of the great pond snail to other androgenic chemicals, *e.g.* organotins, or oestrogenic chemicals, *e.g.* alkylphenols, results in the effects similar to those elicited by steroids, it is an indication of endocrine disruption. To test the hypotheses, a suite of toxicity tests was conducted and the data were analysed using standard statistical as well as toxicokinetic-toxicodynamic (TKTD) modelling approaches.

The results from the toxicity tests on the synthetic steroid hormones 17α-methyltestosterone (MT, androgen) and 17α-ethynyloestradiol (EE2, oestrogen) revealed no effects of these chemicals on various test endpoints related to development, growth, and reproduction of the great pond snail at environmentally relevant concentrations. This suggests that the vertebrate-like steroids are probably not involved in signalling pathways that define the tested performance of snails. Also, no effects were observed when snails were exposed to environmentally relevant concentrations of alkylphenols, which was explained by efficient metabolisation and elimination of these chemicals from the body. Therefore, as MT and EE2 probably do not interfere with the endocrine system of the great pond snail, it was concluded that the two chemicals should not be used as the positive control in toxicity testing for EDs with this species.

Toxicity tests with organotins tributyltin (TBT) and triphenyltin (TPT) demonstrated that the great pond snail is sensitive to low concentrations of these chemicals. Despite the similarity of two organotins with respect to their physico-chemical properties and structural characteristics, they elicited different patterns of effects. In tests with sub-adult and adult snails, sub-lethal endpoints were much more sensitive to TBT than to TPT. When eggs from non-exposed snails were extracted from the jelly-mass and exposed directly to the organotins, the order of toxicity for these two compounds was reversed in comparison to the effects on sub-adults and adults (TPT showed much stronger effects). The reasons for such deviations of the effect patterns of TBT and TPT remained unclear; we speculated that the difference in the alkylation of the two chemicals and/or differences in the expression of certain receptors might be the explanation. Regarding the tests sensitivity, the

most sensitive endpoint for TBT was the frequency of polyembryonic eggs of exposed snails, while for TPT it was the hatching time of eggs directly exposed to the toxicant.

To demonstrate the usefulness of TKTD analysis for putative EDs in the great pond snail, the most suitable test design for this type of analysis needed to be formulated. In a case study for acetone in the great pond snail, the usefulness of different types of data for calibration of a TKTD model was evaluated. Experiments with snails were conducted, in which embryos, sub-adults, and adults were exposed to acetone. Endpoints related to survival, development, growth, and reproduction were monitored over time. Various sets of experimental data were modelled by the DEBkiss model – a model that is based on Dynamic Energy Budget (DEB) theory. Different data sets obtained in the experiments were used for the model calibration. Calibration of the model by different data sets affected the precision rather than the accuracy of the model parameter estimates. The model provided a robust estimation of parameter values, as the calibration upon very different data sets resulted in similar parameter estimates. Data on snail recovery from acetone made surprisingly little improvement on the precision of the parameter estimates. The model calibrated on the most complete data set within the age class best predicted the less complete sets within the same class. Moreover, we could predict both embryonic development and sub-adult and adult life-cycle traits by the same metabolic mode of action (mMoA) – a decrease in assimilation efficiency. This study highlighted that data on embryonic development in treatments can be crucial to selecting a most appropriate mMoA of a toxicant.

DEBkiss was subsequently applied to analyse data from two independent toxicity tests in which sub-adult and adult snails were exposed to a range of TBT concentrations. The model was only slightly modified in order to accommodate differences in temperature and in TBT bioavailability due to different solvent concentrations between the tests. TBT negatively affected survival, growth and reproduction of snails. Analysing the effects by DEBkiss did not require any specific new assumptions about the mMoA of this putative ED. Surprisingly, TBT effects were well described by a mMoA commonly observed for non-EDs: a decrease of the feeding rate. When less energy is available than optimally needed, this leads to a decrease of the growth rate and a smaller ultimate size. The decreased food intake and a smaller body size caused a decreased reproductive output. Therefore, there were no indications that TBT affected reproduction directly.

Finally, recommendations are provided on test design for TKTD analysis as well as for the OECD standardisation of the reproduction test protocol for the great pond snail. The main advantage of a TKTD analysis in respect to the test design is that it allows flexibility. To properly explain effects of toxicants on organisms, growth and reproduction of the same animal cohort should be monitored (preferably individually) over a considerable part of their life cycle, *e.g.* from the juvenile stage well into adulthood. Regarding the future development of the OECD test protocol for the great pond snail, it was recommended to include the monitoring of the egg quality and of the embryonic development as it may increase the sensitivity and relevance of toxicity tests. It would also be invaluable to incorporate size measurements of snails as an additional test endpoint. Chemicals may affect this endpoint directly, which will lead to indirect effects on reproduction of snails, thereby with consequences on population dynamics.

Samenvatting

Hormoonverstorende stoffen (endocriene disruptoren, ED's) zijn lichaamsvreemde chemicaliën die nadelige effecten op organismen hebben doordat ze interacteren met het hormoonstelsel. Deze stoffen zijn bestanddelen van diverse alledaagse producten zoals plastic flessen, voedsel, wasmiddelen en cosmetica, alsmede ingrediënt van een aantal gewasbeschermingsmiddelen en biociden. Door de instelling van het hormoonstelsel te veranderen kunnen ED's effecten hebben op de ontwikkeling, groei en reproductie van organismen. Deze eigenschappen van individuele organismen zijn van cruciaal belang voor de gezondheid van populaties, en dat is het beschermingsdoel van de risicobeoordeling voor aquatische evertebraten (ongewervelde organismen). Er is echter relatief weinig bekend van het hormonale systeem bij evertebraten waardoor het lastig is om ED's te herkennen en hun effecten in te schatten. Het inschatten van de risico's van ED's op populaties wordt tevens belemmerd doordat het gebruik van mechanistische modellen in ecotoxicologisch onderzoek niet breed geaccepteerd is. Dit proefschrift richt zich daarom op het verbeteren van de testmethoden en modellen voor het beoordelen en voorspellen van de effecten van ED's op de poelslak *Lymnaea stagnalis*. Deze zoetwaterslak werd gekozen wegens zijn gevoeligheid voor verschillende stoffen, waaronder enkele ED's. De poelslak is tevens een kandidaatsoort bij de doorlopende standaardisatie van reproductietesten met weekdieren, gecoördineerd door de Organisatie voor Economische Samenwerking en Ontwikkeling (OESO).

In dit proefschrift onderzoek ik de gevoeligheid van de poelslak voor androgene en oestrogene stoffen; stoffen die de ontwikkeling en het onderhoud van respectievelijk de mannelijke en vrouwelijke karakteristieken beïnvloeden. Blootstelling van de poelslak aan steroïdhormonen kan androgene of oestrogene effecten induceren, vergelijkbaar met die in vertebraten en enkele andere waterslakken. Door te volgen hoe de poelslak reageert op steroïdhormonen kan mogelijk de hypothese ondersteund worden dat steroïden een rol spelen in het hormoonstelsel van de poelslak. Als blootstelling aan andere androgene stoffen (zoals organotinverbindingen) of oestrogene stoffen (zoals alkylfenolen) vergelijkbare effecten sorteert als bij steroïden is dat een aanwijzing voor hormoonverstoring. Om deze hypothesen te toetsen werd een reeks van toxiciteitstesten uitgevoerd. De data weren geanalyseerd met behulp van standaard statistische methoden, alsmede met een toxicokinetisch-toxicodynamisch (TKTD) model.

De toxiciteitstesten met de synthetische steroïdhormonen 17α-methyltestosteron (MT, androgeen) en 17α-ethynyloestradiol (EE2, oestrogeen) lieten geen effect zien op de verschillende eindpunten gerelateerd aan ontwikkeling, groei en reproductie van de poelslak bij milieurelevante concentraties. Dit suggereert dat deze steroïden van vertebraten waarschijnlijk niet betrokken zijn bij de hormonale regulatie in de poelslak. Tevens werden er geen effecten gevonden als de slakken werden blootgesteld aan milieurelevante concentraties van alkylfenolen, wat kan worden verklaard door een efficiënt metabolisering en uitscheiding van deze stoffen. Omdat MT en EE2 waarschijnlijk niet interfereren met het hormoonsysteem van de poelslak kunnen deze stoffen niet gebruikt worden als positieve controle voor het testen van ED's met deze diersoort.

Toxiciteitstesten met de organotinverbindingen tributyltin (TBT) en trifenyltin (TPT) toonden aan dat de poelslak gevoelig is voor lage concentraties van deze stoffen. Ondanks dat deze twee stoffen vergelijkbare fysisch-chemische en structurele eigenschappen hebben lieten ze toch een verschillend effectpatroon zien. In testen met jonge en volwassen slakken waren de sub-lethale eindpunten veel gevoeliger voor TBT dan TPT. Deze volgorde in giftigheid was echter omgekeerd voor de embryo's; TPT liet veel sterkere effecten zien (eieren van niet-blootgestelde slakken werden uit hun geleikapsel gehaald en vervolgens direct aan deze organotinverbindingen blootgesteld). De redenen voor de verschillende effectpatronen van TBT en TPT blijven onduidelijk, hoewel we kunnen speculeren over de rol van het verschil in alkylering van de twee stoffen en/of verschillen in de expressie van specifieke receptoren. Het meest gevoelige eindpunt

voor TBT was de frequentie van polyembryonie (meerdere embryo's per ei) terwijl het voor TPT de incubatietijd van de direct blootgestelde eieren was.

Om de bruikbaarheid van TKTD-analyse voor vermeende ED's in de poelslak te demonstreren moest eerst het meest toepasselijke testontwerp voor de poelslak vastgesteld worden. In een casestudie met aceton heb ik verschillende soorten data getoetst op hun bruikbaarheid voor het kalibreren van een TKTD-model. In toxiciteitsexperimenten werden eieren, jonge dieren en volwassen dieren blootgesteld aan aceton. Eindpunten gerelateerd aan overleving, groei en reproductie werden door de tijd gevolgd. De verschillende datasets (in verschillende combinaties) werden geanalyseerd met het DEBkiss model, een model gebaseerd op de theorie van Dynamische EnergieBudgetten (DEB). Het gebruik van verschillende data sets voor modelkalibratie beïnvloedde precisie (breedte van het betrouwbaarheidsinterval van geschatte parameters) meer dan accuratesse (afstand tot de beste parameter schatting). De parameterschatting van het model zijn dus robuust omdat kalibratie op heel verschillende datasets vergelijkbare schattingen opleverde. Het meenemen van 'recovery' (het volgen van dieren in schoon medium na blootgesteld te zijn geweest) leverde verrassend genoeg maar weinig verbetering op in de precisie van de parameterschatting. Het model gekalibreerd op de meest complete dataset binnen een leeftijdscategorie (jonge of volwassen dieren) gaf de beste voorspelling voor minder complete data sets binnen dezelfde categorie. De effecten van aceton op zowel de embryonale ontwikkeling als op de groei en reproductie van jonge en volwassen dieren werden het best beschreven met het metabole werkingsmechanisme (mMoA) 'een afname van de assimilatie-efficiëntie van energie uit voedsel.' De effecten op de embryonale ontwikkeling bleken cruciaal om één enkel werkingsmechanisme te selecteren uit een aantal mogelijk opties.

DEBkiss werd eveneens toegepast om de data van twee onafhankelijke toxiciteitstesten met TBT te analyseren: een test met jonge slakken en een aparte test met volwassen slakken. Het model kon licht worden aangepast om de verschillen tussen de twee tests te ondervangen: een verschil in testtemperatuur en een verschil in de biobeschikbaarheid van TBT veroorzaakt door een andere concentratie oplosmiddel. TBT had een negatief effect op overleving, groei en reproductie van de poelslakken. Ondanks het feit dat TBT een vermeende ED is waren er geen specifieke nieuwe aannames over het werkingsmechanisme nodig om de effecten te verklaren; een algemene mMoA voor niet-ED's, 'afname van de eetsnelheid', was voldoende. Als een dier minder energie beschikbaar heeft dan optimaal noodzakelijk is leidt dat tot een afname van de groeisnelheid en een kleinere uiteindelijke lichaamsgrootte. De afname van de eetsnelheid gekoppeld aan de kleinere lichaamsgrootte veroorzaakt een afname in de reproductie. Er was dus geen indicatie dat TBT de reproductie direct beïnvloedde.

Tenslotte geef ik een serie aanbevelingen omtrent testontwerp voor TKTD-analyse en voor de OESO-standaardisering van het reproductie testprotocol voor de poelslak. Het belangrijkste voordeel van een TKTD analyse voor het testontwerp is dat het een grote mate van flexibiliteit toestaat. Om de effecten van toxische stoffen op organismen goed te verklaren dienen groei en reproductie van één en hetzelfde cohort dieren (liefst individueel) gevolgd te worden over een aanzienlijk deel van hun levenscyclus (vanaf het juveniele stadium tot goed in het volwassen stadium). Met betrekking tot de toekomstige ontwikkeling van het OESO-testprotocol voor de poelslak: de gevoeligheid en relevantie van deze toxiciteitstest kan vergroot worden door tevens de eikwaliteit en de embryonale ontwikkeling te volgen. Verder is het uiterst waardevol om de grootte van de dieren te volgen als additioneel eindpunt (schelplengte over de tijd alsmede drooggewicht van de zachte delen aan het einde van de test). Chemicaliën kunnen dit eindpunt direct beïnvloeden, wat leidt tot indirecte effecten op reproductie en daardoor met gevolgen voor de populatiedynamiek.

Résumé étendu

Chapitre 1 : Introduction

Le système endocrinien est impliqué dans le maintien de l'homéostasie des animaux et régule les processus physiologiques relatifs à leur développement, leur croissance et leur reproduction. Dans ce contexte, un perturbateur endocrinien se définit comme une substance exogène capable d'altérer le fonctionnement du système endocrinien, avec pour conséquence l'apparition d'effets chez les individus exposés, effets pouvant aboutir à des conséquences sur la population. L'apparition d'imposex (développement de tissus reproducteur males chez des femelles, ou inversement) est un exemple bien connu de perturbation endocrinienne chez les mollusques, dû à l'exposition au tributylétain (TBT).

L'évaluation du risque des perturbateurs endocriniens nécessite une attention particulière, puisque ces composés sont capables d'engendrer des effets à de très faibles concentrations et ce au moment de l'exposition, ou plus tard au cours de la vie des animaux, voir sur leur descendance. Les courbes concentration - réponse associées peuvent être différentes de la courbe en S classiquement observée en écotoxicologie (*e.g.* courbe en U ou en U-inversé). Leur analyse demande donc d'avoir recours à des méthodes de bioessais et d'analyse de données particulières. La réglementation portant sur l'évaluation du risque de ces composées est elle aussi spécifique. Elle requiert le développement de critères permettant d'identifier les substances perturbant le système endocrinien, ainsi que de méthodes fiables pour analyser leurs effets non-intentionnels sur la faune. Dans ce contexte, la capacité des molécules testées à interagir avec le système endocrinien est généralement évaluée *via* une batterie de tests *in vitro*, tandis que leur capacité à engendrer des effets sur les individus requiert des bioessais *in vivo*. Afin de démontrer la caractère perturbateur endocrinien d'une molécule, il faut établir des relations de cause à effet entre le mode d'action du produit déterminé *in vitro* et ses effets mesurés *in vivo*. Les tests standardisés pouvant être utilisés comme support pour la réglementation sont définis dans le document guide de l'Organisation de coopération et de développement économique (OCDE) N° 150 et résumés en figure 1.2 (p 4 du manuscrit). Parmi les invertébrés, les mollusques s'avèrent plus sensibles à certains types de perturbations endocriniennes que les espèces standards (chironomes, daphnies) : cela explique pourquoi les tests standardisés disponibles actuellement n'avaient pas permis de prévoir les effets du TBT. Par conséquent, l'OCDE soutient depuis 2011 un programme de développement de bioessais standards sur mollusques, visant à évaluer la toxicité des xénobiotiques (y compris des perturbateurs endocriniens) sur les performances individuelles, et en particulier sur la reproduction. La lymnée des étangs (*Lymnaea stagnalis*), un gastéropode d'eau douce hermaphrodite simultané, figure parmi les espèces standards pour conduire ces bioessais de reprotoxicité. Dans ce contexte, cet organisme a été choisi comme modèle biologique pour cette thèse.

L'endocrinologie et le contrôle endocrinien de la croissance et de la reproduction est bien connu chez cette espèce. Ces connaissances peuvent servir de contexte théorique pour la mise en place de relations de cause à effet entre les mécanismes d'action des xénobiotiques et leurs effets à l'échelle de l'individu. Ces relations peuvent être ensuite formalisées sous forme d'un diagramme conceptuel de type « adverse outcome pathways » ou AOP (figure 6.2 p 84 du manuscrit). Cependant de nombreuses informations nous manquent encore pour établir ces AOP dans le cadre de l'étude de la perturbation endocrinienne. En particulier, le mécanisme initiateur de la réponse au xénobiotique est difficile à identifier. Ceci est lié au fait que les composés perturbateurs endocriniens sont généralement identifiés sur la base de leur capacité d'interaction avec des récepteurs aux hormones stéroïdiennes utilisées dans le système endocrine chez les vertébrés. Pour l'instant, seules certains récepteurs et certaines hormones stéroïdes ont été identifiés chez la lymnée. Par ailleurs, nous ignorons leur rôle dans le contrôle hormonal de la reproduction. Dans ce contexte, les mécanismes initiateurs de toxicité et la cascade de réactions physiologiques générant l'apparition d'effet

toxiques chez la lymnée nous sont généralement inconnus. Afin de les élucider, la démarche scientifique communément utilisée consiste à transposer les connaissances disponibles chez les vertébrés au contexte endocrinien de la lymnée, et à vérifier leur pertinence dans ce contexte. Cependant, la simple transposition des mécanismes peut s'avérer infructueuse : il nous faut une donc développer autre méthode d'investigation de ces mécanismes.

Lorsque la cible du toxique et la cascade de réactions physiologiques générant des effets sur les individus sont connus, il nous faut ensuite établir des relations quantitatives entre ces éléments de cet AOP. En effet, les relations quantitatives entre les niveaux sub-individu, individu, et population sont nécessaire à l'analyse des résultats de bioessais qui sous-tendent l'évaluation du risque du xénobiotique. Les modèles d'effet mécanistes, qui prennent en compte à la fois l'information sur la physiologie des individus et sur leur performances individuelles sont les outils les plus adaptés pour obtenir ces relations quantitatives, qui serviront ensuite à prédire les effets du xénobiotique étudié sur les individus et à extrapoler aux possibles effets populationnels.

Parmi les modèles mécanistes d'effet utilisés en écotoxicologie, les modèles de toxicocinétique-toxicodynamique (TK-TD) sont les mieux adaptés à l'analyse des données de bioessais dans le contexte de l'évaluation du risque. En se basant sur les propriétés physico-chimiques des xénobiotiques et la biologie des organismes étudiés, ces modèles mathématiques décrivent les processus physiologiques impliqués dans (i) l'absorption/l'élimination du produit par l'organisme et (ii) sa réponse à la présence du xénobiotique dans ses tissus. Cette dimension mécaniste permet d'intégrer l'ensemble des connaissances relatives à la toxicité des xénobiotiques, à la physiologie et à la sensibilité de l'espèce étudiée et à ses capacités de récupération, apportant ainsi un fort réalisme biologique à l'évaluation du risque d'un xénobiotique.

Le modèle de budget énergétique dynamique (DEB) est un modèle TK-TD générique, basé sur la physiologie des organismes. Les hypothèses du modèle reposent sur l'observation suivante: l'élimination du xénobiotique présent dans le corps de l'animal, la mise en œuvre de mécanismes de défense contre ce produit et la réparation des dégâts causés par ce produit ont un coût énergétique important. Ce surcoût énergétique mobilise en priorité l'énergie disponible chez l'individu exposé, ce qui induit une diminution de la quantité d'énergie allouée aux grandes fonctions physiologiques (telles que la croissance, le développement, la maturation sexuelle et la reproduction) et des modifications des règles d'allocation de l'énergie à ces fonctions (figure 1.3, p 5 du manuscrit). Ces dérèglements de l'allocation énergétique sont à l'origine d'une modification des performances individuelles.

Comme en attestent plus de 70 publications, ce modèle a été validé pour de nombreux xénobiotiques chez diverses espèces de vertébrés et d'invertébrés, si bien que les sorties du modèle (EC_x, concentration sans effet) sont officiellement utilisables en évaluation du risque depuis 2006.

Le modèle DEB est intéressant pour l'analyse des données de bioessais dans le contexte de l'évaluation du risque des perturbateurs endocriniens, car c'est la seule approche qui permet de mettre en relation les effets observés sur l'ensemble des performances individuelles susceptibles d'être affectées par les perturbateurs endocriniens (croissance, développement, maturité sexuelle, fécondité, qualité de la descendance) dans un même modèle d'effet. Cette approche intégratrice du cycle de vie a été très peu utilisée jusqu'à présent pour l'étude des perturbateurs endocriniens (PE) malgré sa pertinence écotoxicologique (seulement deux études publiées). En effet, les PE agissent non seulement sur la valeur des traits de vie des animaux exposés, mais aussi sur les relations de compensation ("trade-off") entre ces traits, qui sont sous contrôle hormonal et qui déterminent comment les effets individuels se répercutent au niveau des populations. En prenant en compte ces relations de compensation, le modèle DEB permet de fournir des estimations de performances individuelles plus pertinentes par rapport à la biologie de l'espèce étudiée. Ces prédictions permettent de calculer des concentrations sans effet réalistes sur les populations, répondant ainsi aux objectifs de protection fixés par la réglementation, mais non encore atteints pour les perturbateurs endocriniens.

Par ailleurs, ce modèle permet de prédire les effets du xénobiotique étudié sur les performances individuelles au cours du temps, en fonction du profil d'exposition. Ce pouvoir prédictif est un atout majeur qui justifie l'utilisation de ces modèles en évaluation du risque. En effet, les modèles DEB peuvent être utilisés pour extrapoler les résultats des tests de toxicité entre espèces, entre toxiques et entre scenarios d'exposition. Les autorités en charge de l'évaluation du risque attendent beaucoup de ces modèles, car leur utilisation (i) permet de diminuer le nombre et les coûts des tests de toxicité à réaliser pour l'évaluation du risque a priori et (ii) fournit des informations utiles pour définir les mesures de réduction des risques propres aux usages d'un produit.

Une formulation générique simplifiée du modèle DEB, appelée DEBkiss, a été développée et validée chez la lymnée en conditions de laboratoire (Thèse E. Zimmer, conduite en collaboration avec le présent travail de thèse). Ce modèle générique repose sur l'hypothèse selon laquelle le produit agit sur une (ou quelques) fonctions physiologiques majeures pour l'organisme, provoquant une modification de la quantité d'énergie allouée à cette fonction. Certaines de ces fonctions physiologiques sont sous contrôle hormonal. Ainsi, il est probable que les perturbateurs endocriniens affectent ces fonctions. Par exemple, chez les bivalves, les xéno-œstrogènes ont une action directe sur la reproduction (*e.g.* modification de la sex-ratio, de l'indice gonado-somatique, de la synthèse de vitellogénine) et une action indirecte sur les processus immunitaires sous contrôle hormonal (*e.g.* modification de la structure et de la fonction des hémocytes). Si certaines de ces perturbations (*e.g.* diminution de la synthèse de vitellogénine) entrainent des modifications du budget énergétique, d'autres (*e.g.* altérations du système immunitaire) ne sont pas directement liées à la gestion de l'énergie, bien qu'elles aient des répercussions sur le budget énergétique (*e.g.* augmentation de la quantité d'énergie nécessaire pour maintenir le fonctionnement du système immunitaire). Pour l'instant, nous ignorons si le modèle DEBkiss générique, très simple au regard de la physiologie des organismes, permet d'appréhender les mécanismes complexes impliqués dans la réponse aux perturbateurs endocriniens ou si au contraire, des hypothèses toxicologiques supplémentaires relatives au mode d'action du xénobiotique devront être ajoutées afin de prédire les effets sur le cycle de vie des individus.

Dans ce contexte, ce travail de thèse vise à contribuer à la mise au point de méthodes de tests de toxicité sur la lymnée de étangs, ainsi que de modèles mécanistes d'effets permettant d'analyser les données issues de ces tests dans le cadre de l'évaluation du risque des xénobiotiques.

Les objectifs spécifiques sont les suivants :

- à partir de la méthode de bioessais en cours de standardisation par l'OCDE, nous souhaitons développer des protocoles de tests de toxicité mieux adaptés à l'obtention des données nécessaires à l'analyse des effets par des modèles mécanistes de type DEBkiss.
- nous souhaitons appliquer ces protocoles à l'étude des réponses de la lymnée à différents perturbateurs endocriniens, afin d'évaluer leur toxicité chez cette espèce.
- les données issues de ces tests de reprotoxicité seront analysés avec le modèle DEBkiss afin (i) de tester les capacités de ce modèle générique à prédire les réponses des lymnées et de le modifier si nécessaire et (ii) d'investiguer les mécanismes d'action des xénobiotiques choisis chez la lymnée.

Le manuscrit de thèse présentant ces méthodes et résultats s'organise comme suit.

Le second chapitre s'intéresse aux effets engendrés par des xénobiotiques supposés avoir des propriétés androgéniques (sur la base des résultats de tests chez les vertébrés), sur la survie, la croissance et la reproduction des lymnées. Dans ce contexte, nous comparons les effets de deux molécules de structures chimiques proches, le tributylétain et le triphénylétain. Ces effets sont mesurés à l'aide de méthodes standards : bioessais en laboratoire conduits conformément à la méthode en cours de standardisation par l'OCDE et analyse des données grâce aux méthodes de statistique descriptives recommandées par l'OCDE.

Le troisième chapitre présente une approche d'optimisation des méthodes de bioessais utilisées au chapitre précédant en vue de l'analyse des données par le modèle DEBkiss. Le mode d'action du

produit important peu pour cet exercice, nous avons utilisé l'acétone comme xénobiotique modèle. Nous avons étudié l'influence de nombre et du type de données collectées sur le processus de calibration du modèle et les conséquences sur les sorties du modèle, et ce afin de déterminer le ratio cout-bénéfice de différents types de plan expérimentaux. Nous utilisé un jeu de donné indépendant (effets de l'acétone sur les embryons de lymnée) afin de valider notre modèle.

Le quatrième chapitre vise à évaluer l'applicabilité du modèle générique DEBkiss (calibré selon les méthodes présentées au chapitre 3) afin de prédire les effets du tributylétain (TBT) sur le cycle de vie de la lymnée. Pour ce faire, des tests de toxicité sur embryons, juvéniles et adultes ont été réalisés. Nous utilisons aussi le modèle DEBkiss afin d'étudier le mode d'action du TBT sur la lymnée.

Le chapitre cinq s'intéresse aux effets de différents xénobiotiques supposés avoir une action œstrogénique chez les vertébrés (17α-ethyniloestradiol et alkylphénols) sur la survie, la croissance et la reproduction de la lymnée. Là encore, nous utilisons différents plans expérimentaux et critères d'effets, et discutons de leur capacité à mettre en évidence des effets sur les embryons, les juvéniles et les adultes de lymnée. Nous confrontons ces résultats aux connaissances disponibles quant aux effets des composés œstrogéno-mimétiques chez les mollusques.

La discussion aborde tout d'abord des questions relatives aux effets des perturbateurs endocriniens chez les lymnées, et aux possibles mécanismes expliquant ces effets. Nous discutons ensuite des avantages et des limites du modèle DEBkiss pour l'analyse des données écotoxicologiques, en nous focalisant sur leur contribution à l'apport de connaissance sur les mécanismes d'effet des perturbateurs endocriniens. Nous abordons ensuite la question des méthodes de tests de toxicité pour l'évaluation du risque des perturbateurs endocriniens, en se focalisant surtout sur le choix des critères d'effets biologiques. Nous concluons sur les perspectives de recherche qui permettrait d'améliorer notre approche qualitative et quantitative des effets toxiques dans le cadre de la construction d'un AOP, à l'aide de bioessais appropriés et du modèle DEBkiss.

Chapitre 2 : Etude des effets du tributylétain et du triphénylétain sur la lymnée

Ce chapitre présente une étude écotoxicologique classique visant à déterminer les effets du tributylétain (TBT) et du triphénylétain (TPT) sur la lymnée à l'aide de méthodes inspirées des protocoles de bioessais et d'analyse statistique de données en cours de standardisation par l'OCDE. Le TBT a été choisi car il constitue un exemple emblématique de perturbateur endocrinien chez les mollusques. Il a été étudié tout particulièrement chez des mollusques marins dont les espèces présentent un mode de reproduction gonochorique (à sexe séparé). Chez ces espèces, il génère des problèmes de morphologie des gonades (imposex) ainsi que des réductions de la fécondité. Ces effets sont supposés être liés à ses propriétés androgéniques, celle-ci ayant été démontrées chez les vertébrés. Ses effets sur la reproduction de la lymnée, un animal d'eau douce à reproduction hermaphrodite, n'ont encore été que peu étudiés. Sur la base des résultats observés chez les autres mollusques, nous attendons des effets de ce produit à de faibles concentrations, ainsi que des courbes dose-réponse en U-inversé. Le TPT a été choisi car il possède une structure chimique proche de celle du TBT, et un mode d'action androgénique comparable chez les vertébrés. Dans ce contexte, nous attendons des effets qualitativement similaires à ceux du TBT.

Afin de tester ces hypothèses, nous avons conduit en parallèle deux tests de toxicité de 21 jours sur des lymnées adultes. Les lymnées utilisées proviennent d'un élevage en conditions contrôlées maintenu à l'INRA de Rennes, et utilisant la souche Renilys®.

Les lymnées ont une durée de vie d'un à deux ans en laboratoire. Le cycle de vie se compose de trois phases. Les embryons sont rassemblés par groupes de 50 à 150 individus dans des pontes, déposées par les parents sur un support solide (paroi de l'aquarium en laboratoire, plantes ou substrat en milieu naturel). Les nouveau-nés ont une taille d'1 mm environ et se nourrissent

principalement de biofilm présent sur les parois des aquariums. Les juvéniles comme les adultes sont omnivores et se nourrissent principalement de végétaux vivants ou en décomposition. Lors des tests de toxicité, les animaux sont nourris chaque jour à l'aide de laitue. Les juvéniles atteignent la maturité sexuelle à la taille de 2 cm environ. Dès lors, ils se reproduisent régulièrement jusqu'à la fin de leur vie. La reproduction se fait préférentiellement par fécondation croisée, bien que des cas d'autofécondation soient observés en milieu naturel en l'absence prolongée de congénères.

Les lymnées adultes utilisées pour les tests de toxicité sont d'âge et de taille homogènes (environ 4 mois et 22,5 mm dans notre étude). Ces animaux sont prélevés dans l'élevage et acclimatés au milieu de test durant 2 jours avant de procéder aux expositions. Les concentrations en TBT et TPT choisies dans cette étude sont les suivantes : 19, 43, 94, 197, et 473 ng Sn/L de TBT, et 45, 74, 187, 265, et 590 ng Sn/L de TPT. Ces contaminants hydrophobes sont ajoutés dans l'eau à l'aide de 100 µL/L d'acétone. Pour chacune de ces concentrations, nous mettons en place six réplicats de cinq individus. Un nombre identique d'animaux et de réplicats est utilisé pour les témoins eau et acétone. Le milieu d'exposition est renouvelé deux fois par semaine. Le nombre d'animaux vivants, ainsi que le nombre de pontes produites par réplicat est évalué chaque jour. Les pontes sont collectées et le nombre d'œufs par ponte est déterminé suite à l'observation de la ponte à la loupe binoculaire. Cette observation nous permet par ailleurs de compter le nombre d'embryons malformés par ponte et d'identifier le type de malformation (œufs sans albumen, œufs avec albumen atrophiés, œufs « vides » : sans embryon, œufs polyembryonnés : avec plusieurs embryon par œuf). La taille des individus est mesurée chaque semaine. Les animaux sont maintenus à une température de 20 °C environ et sous une photopériode 14 : 10 jour/nuit. Ils sont nourris *ad libitum* chaque jour ouvré.

Les données ainsi obtenues sont analysées à l'aide des tests statistiques et des modèles de régression recommandés par l'OCDE pour déterminer la concentration sans effet observé (NOEC) et la concentration affectant x% des animaux exposés (EC_x). Ainsi, les différences significatives à $p = 0,05$ entre témoins et traités pour la survie, la taille, le nombre cumulé de pontes et d'œufs produit par individu sont identifiés à l'aide des tests de Kruskall–Wallis tests, suivis de tests post hoc de Dunn's tests ou de Dunnet's. Les différences significatives entre témoins et traités pour la fréquence d'œufs malformés sont identifiés à l'aide du test de Mann–Whitney. Lorsque des effets significatifs sont observés entre témoins, seul le témoin solvant est utilisé pour les tests statistiques: sinon, les données témoins eau et solvant sont rassemblées. Lorsque des effets significatifs sont observés entre témoins et traités, la courbe concentration-réponse est modélisée par un modèle logistique à trois paramètres, permettant de calculer les valeurs d'EC_{50}, d'EC_{10} et leur intervalle de confiance à 95%.

Seule l'exposition à la plus forte concentration testée en TPT a engendré des effets significatifs sur la survie des lymnées exposées (LC_{50} = 436,1 [IC95% : 308,1 – 433,6] ng Sn/L). L'exposition aux organoétains a induit une fragilisation de la coquille des animaux exposés à plus de 19 ng Sn/L de TBT et à toutes les concentrations de TPT testées. Par ailleurs, le TBT a induit une diminution de la croissance pour des concentrations d'exposition supérieures à 94 ng Sn/L TBT. Le TPT n'a pas provoqué d'effet significatif sur la croissance des lymnées exposées. Les deux composés ont induit une réduction de la fréquence de pontes par les lymnées, à partir de 94 ng Sn/L de TBT (EC_{50} = 118,3 [IC95% : 99,7 – 171,9] ng Sn/L) et de 265 ng Sn/L TPT (EC_{50} = 264,1 [IC95% : 258,5–280,5] ng Sn/L).

Chapitre 3 : Optimisation des plans d'expériences des tests de toxicité en vue de l'analyse des données par des modèles d'effets mécanistes

La standardisation des tests de toxicité assure la reproductibilité des résultats obtenus lors de l'exposition d'une espèce standard à un xénobiotique donné. Pour cette espèce, cette standardisation facilite aussi la comparaison entre les résultats de tests obtenus pour différents xénobiotiques. Les lignes directrices OCDE qui définissent les protocoles standardisés de test signalent que ces protocoles doivent être adaptés en fonction du type d'analyse utilisée pour interpréter les données.

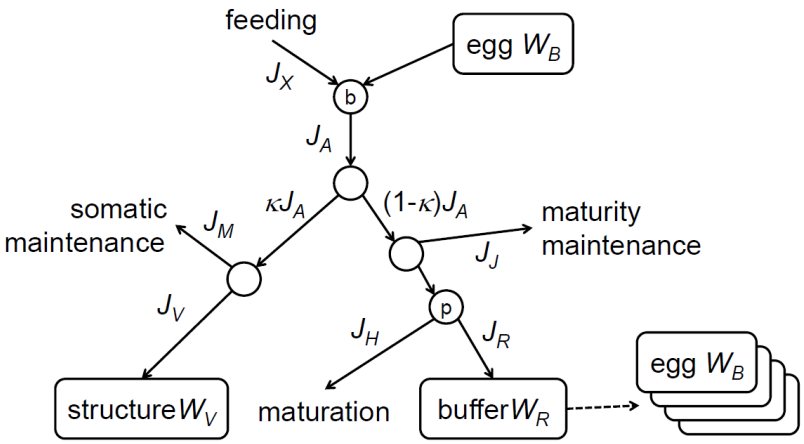
Ainsi, afin de calculer une NOEC, il est recommandé de mettre l'accent sur le nombre de réplicats étudiés afin d'augmenter la puissance des tests d'hypothèses. Afin de calculer une EC_x, il est recommandé de mettre l'accent sur le nombre de concentrations testées, afin d'obtenir une courbe concentration réponse se prêtant à l'analyse par régression. Cependant, les lignes directrices OCDE ne donnent pas de consignes pour l'optimisation du plan d'expérience lorsque nous souhaitons analyser les données à l'aide d'un modèle d'effet mécaniste. Dans ce chapitre, nous explorons différents types de plan d'expérience afin d'identifier celui qui permet une utilisation optimale de ce type de modèles.

Pour ce faire, nous avons réalisé des tests de reprotoxicité sur la lymnée exposée à un narcotique (l'acétone). Nous avons choisi ce composé car il est utilisé dans les autres chapitres (ainsi que dans les tests de toxicité standard en général) en tant que solvant, permettant de dissoudre les xénobiotiques dans le milieu aqueux d'exposition. Afin d'éviter un biais lors de l'étude de la toxicité de ces xénobiotiques, il est important de savoir à partir de quelle concentration le solvant peut induire des effets toxiques.

Nous avons réalisés cinq tests de toxicité avec un plan expérimental différent :

- *Exposition de juvéniles isolés et d'adultes isolés, sans dépuration.* Ces deux tests de 56 jours impliquent le suivi de 25 animaux témoins et de 5 animaux par concentration en acétone testée. Nous avons utilisé une gamme de concentration de 0,1 mL/L à 18 mL/L. Les juvéniles deviennent sexuellement matures durant le test. Les animaux capables de se reproduire sont mis en couple pendant 8h, et ce deux fois par semaine, afin de permettre la copulation. Les variables biologiques suivantes ont été étudiées : survie et nombre de pontes produites par individu (évalués tous les jours), taille de la coquille (évaluée une fois par semaine), poids sec des pontes et poids secs des animaux (évalués après la ponte et à la fin du test, respectivement).
- *Exposition de juvéniles isolés et d'adultes isolés, avec dépuration.* Ces tests ont été conduits sur quatre juvéniles et quatre adultes isolés, selon le même protocole que précédemment. Après 14 jours d'exposition, les animaux ont été transférés dans un milieu ne contenant pas d'acétone jusqu'à la fin de l'expérience (56 jours).
- *Exposition des embryons.* Après avoir prélevé des pontes dans notre élevage, les œufs ont été extraits. Nous avons exposé 12 œufs par concentration testée (dans la gamme 0,02 mL/L à 18 mL/L) pendant 21 jours. Les variables biologiques suivantes ont été étudiées : taux d'éclosion moyen, taille des nouveau-nés (évaluée à la fin de l'expérience), durée du développement embryonnaire (sur la base d'un suivi journalier).

Les données obtenues ont été analysées à l'aide du modèle DEBkiss. Les hypothèses du modèle sont les suivantes. Les performances individuelles sont reliées entre elle *via* l'allocation différentielle de l'énergie, comme présenté ci-dessous :



Dans ce digramme (tiré de Barsi *et al.* 2014), la « structure » représente la biomasse de l'animal : corps mou, à l'exclusion de la coquille et des réserves convenues dans la glande à albumen (celles-ci sont représentées dans le compartiment « buffer »). Ces réserves sont transformées en œufs (compartiment « egg ») lors de la reproduction. Les œufs utilisent leurs réserves pour se développer et éclore (cercle « b » pour « birth »). Les juvéniles et les adultes utilisent la nourriture présente *ad libitum* dans le milieu d'expérience (le taux d'alimentation est nommé *Jx*). Les animaux utilisent une partie (κJ_A) de l'énergie assimilée pour assurer la maintenance de leurs tissus (« somatic maintenance », flux J_M) et pour grandir (flux J_v). La fraction d'énergie restante (1 - κJ_A) est utilisée pour le développement sexuel (flux J_H) aboutissant à la puberté (cercle « *p* »), la maintenance des gonades (flux J_J) et la mise en réserve d'énergie dans le « buffer » afin de former les œufs à la prochaine reproduction (flux $J_{R)}$. Ces hypothèses forment le modèle de la physiologie de la lymnée en conditions témoins. Des hypothèses supplémentaires sont nécessaires en milieu contaminé. L'absorption du xénobiotique depuis l'eau d'exposition vers les tissus de l'animal est prédite suivant un modèle de toxicocinétique de premier ordre à un compartiment. Les effets toxiques ne surviennent que lorsqu'une concentration interne seuil (la NEC : « no-effect concentration ») est excédée : ils sont alors proportionnels à l'augmentation de la concentration interne en xénobiotique au cours du temps. Les effets s'exercent en modifiant l'allocation d'énergie aux différentes fonctions physiologiques : la maintenance et la réparation des tissus est privilégiée, au détriment de la croissance et / ou de la reproduction. Il existe différents scenarios, dans lesquels différentes fonctions physiologiques majeures sont affectées par le xénobiotique (*e.g.* maintenance, assimilation de la nourriture, croissance, reproduction). Ces différents scénarios correspondent à différent « modes d'action » (ou MoA) dans le modèle DEBkiss. Si les couts de maintenance nécessaires à la réparation des dommages induits par le xénobiotique sont trop élevés, alors l'animal meurt. Les équations différentielles qui sous-tendent ce modèle sont présentées dans Barsi *et al.* 2014.

Une démarche progressive a été utilisée afin de calibrer ce modèle à partir des différents jeux de données disponibles. Le modèle a tout d'abord été calibré en utilisant les données témoins des expériences sur les adultes et les juvéniles, soit séparément, soit en combinaison. Ensuite, le modèle a été calibré en présence d'acétone, en utilisant les données des tests de toxicité (avec ou sans dépuration) sur les adultes et les juvéniles, soit séparément, soit en combinaison. Au total, neuf jeux de donnée de complexité croissante ont été utilisés pour la calibration du modèle. Par ailleurs, le modèle a été calibré pour chacun les différents MoA mentionnés ci-dessus. Les sorties des différents modèles, calibrés selon les différents jeux de données et MoA, ont enfin été comparées afin d'identifier (i) le jeu de données optimisant la calibration du modèle et (ii) le mode d'action de l'acétone chez les lymnées exposées. Le jeu de donnée sur les embryons n'a pas été utilisé pour la calibration, mais pour la validation du modèle. Cette validation consiste dans notre cas à vérifier si le modèle calibré sur la base des données juvéniles et adultes est capable de prédire le développement embryonnaire et la taille des embryons à l'éclosion.

L'utilisation des jeux de données témoins pour 25 juvéniles et adultes suivis pendant 56 jours a permis une bonne calibration du modèle : en effet les faibles intervalles de confiances obtenus pour les valeurs des paramètres du modèle confirment que ces paramètres ont été correctement estimés. Le modèle ainsi paramétré permet de décrire de façon fiable la croissance et le développement des juvéniles, mais aussi la croissance et la reproduction des adultes. Ceci confirme que le modèle DEBkiss permet d'appréhender différentes étapes du cycle de vie de l'animal (ici la phase juvénile et adulte) à l'aide du même jeu de paramètres. Pour certains paramètres (*e.g.* taille à la puberté), les valeurs obtenues dans cette étude ne correspondent pas tout à fait les résultats d'études précédentes en conditions témoins avec la lymnée, mais elle reste du même ordre de grandeur. Ces différences sont attribuées à des différences entre les conditions expérimentales maintenues pendant dans les différentes études, sans que celles-ci n'aient été clairement identifiées.

Le modèle DEBkiss a aussi permis de représenter correctement les effets de l'acétone pour la plupart des variables biologiques suivies, mis à part la survie et le poids sec des animaux : ceci

s'explique par un plus faible nombre de données disponibles pour ces deux critères d'effet, pour lesquelles ne disposons que d'une seule mesure au cours du test. Le modèle DEBkiss permet de mettre en évidence des effets subtils, tel que le phénomène d'hormèse à la plus faible concentration testée, ou le phénomène de perte graduelle de poids au cours du test aux concentrations les plus élevées. Cette perte de poids est décrite de façon correcte chez les adultes, mais elle est sous-estimée chez les juvéniles : cela suggère qu'il sera nécessaire de revoir les hypothèses relatives à la perte de poids dans notre modèle. Les modèles supposant une action de l'acétone sur l'assimilation de la nourriture ou sur le cout de la maintenance des tissus nous fournissent des ajustements de qualité comparables. Il n'est donc pas possible de décider du mode d'action de l'acétone chez la lymnée sur la base de ces seules données (juvéniles et adultes). En revanche, le modèle permet de décrire de façon fiable la restauration des performances individuelles de tous les animaux durant la phase de dépuration en milieu non contaminé. Cela confirme que les effets de l'acétone sont réversibles aux concentrations et durée d'exposition testées. Par ailleurs, la concentration en acétone sans effet sur la lymnée a été évaluée à 13,5 mL/L (IC95% : 12,6–13,8). Cette valeur est très nettement supérieure aux concentrations en acétone utilisées pour dissoudre les xénobiotiques lors des tests de toxicité (10, 20, ou 100 µL/L). Au vu de ces résultats, il est peu probable que l'utilisation d'acétone comme solvant organique au cours des tests de toxicité présentés dans les chapitres suivants ait aggravé les effets des xénobiotiques étudiés.

Concernant les aspects calibration, il s'avère que les neuf jeux de données utilisés ont donné lieu à valeurs de paramètres comparables, et ce pour la plupart des paramètres. Ceci témoigne de la robustesse de la méthode utilisée pour calibrer le modèle (maximum de vraisemblance). Le choix du jeu de donnée influence cependant la précision de l'estimation, représentée par l'étendue de l'intervalle de confiance à 95%. Comme attendu, la meilleure précision est obtenue lorsque l'ensemble des données disponibles est utilisé pour la calibration du modèle. Seul le paramètre impliqué dans la modélisation de la perte de poids des animaux a obtenu des valeurs différentes en fonction du jeu de donnée utilisé pour la calibration. Ceci est coïncide avec le fait que le modèle sous-estime la perte de poids des juvéniles par rapport aux données).

Le modèle calibré à l'aide d'un jeu de donné particulier a ensuite été utilisé afin de « prédire » les données obtenues dans les autres expériences. Il s'avère que le modèle calibré avec l'ensemble des données permet de reproduire de façon correcte n'importe lequel des jeux de données isolé. Ceci ne s'applique pas aux modèles calibrés avec seulement une partie des données : les prédictions du modèle semblent très sensibles aux valeurs des paramètres. Afin d'optimiser la fiabilité des prédictions du modèle, il est donc recommandé d'utiliser l'ensemble des données disponibles pour la calibration. Par ailleurs, le modèle calibré à partir de l'ensemble des données adulte et juvénile a été utilisé pour prédire les performances des embryons témoins, ainsi que les effets de l'acétone sur les embryons. Le patron de développement des embryons témoins est reproduit de façon correcte sur l'aspect qualitatif, mais de façon incorrecte sur l'aspect quantitatif : le modèle prédit un développement embryonnaire trop rapide et une taille à l'éclosion trop élevée. Ces différence sont probablement liés au fait que le taux d'assimilation de la nourriture chez les embryons (qui assimilent directement les réserves de l'œuf) est différent de celui des juvéniles et adultes (qui se nourrissent des aliments fournis par l'expérimentateur et doivent les digérer) : cette différence n'a pas été prise en compte dans le modèle. Des facteurs correctifs ont été introduits dans le modèle témoins avant de procéder aux prédictions en milieu contaminés. Ainsi, les effets de l'acétone sur le développement des embryons ont pu être prédits correctement par le modèle. L'analyse des donnée obtenues chez les embryons a par ailleurs permis dans déterminer le mode d'action le probable pour l'acétone chez la lymnée : il s'agit d'une diminution de l'efficacité d'assimilation de la nourriture.

En conclusion, ce chapitre a permis de développer un modèle mécaniste du cycle de vie de la lymnée en conditions témoins et toxiques. Ces résultats permettront de proposer des recommandations en termes de plan expérimental pour les bioessais avec la lymnée dans le cas où les données sont analysées à l'aide d'un modèle d'effet mécaniste tel que DEBkiss (voir

discussion). Ce chapitre a par ailleurs permis de démontrer l'innocuité de l'acétone aux concentrations généralement utilisées dans les bioessais afin de dissoudre les xénobiotiques.

Chapitre 4 : Etude approfondie des effets du tributylétain et de son mécanisme d'action toxique chez la lymnée grâce à la modélisation

Le chapitre 1 nous a permis d'évaluer les effets du tributylétain (TBT) sur la reproduction de la lymnée en utilisant une approche classique en écotoxicologie. Cette approche s'est focalisée sur l'étude de la réponse d'adultes exposés au xénobiotique pendant une courte période: elle ne permet donc pas d'appréhender les réponses sur l'ensemble du cycle de vie de l'animal. Par ailleurs, les données obtenues étaient insuffisantes pour aborder solidement la question du mode d'action du TBT chez la lymnée. Dans ce chapitre, nous utilisons les méthodes expérimentales et le modèle DEBkiss présentés au chapitre 3 à fin d'explorer en détail les effets du TBT et son mécanisme d'action chez les embryons, les juvéniles et les adultes de lymnées. Ce travail vise notamment à vérifier si le TBT possède ou non des propriétés de perturbateur endocrinien pour cette espèce.

Pour ce faire, nous avons réalisé trois tests de toxicité indépendants selon des plans expérimentaux inspirés des résultats des chapitres 2 et 3. Le premier test de toxicité a consisté en une exposition de 35 jours de juvéniles (4 individus par traitement, exposés en isolation) à des concentrations comprises entre 11 et 2743 ng Sn/L de TBT. Dans le second test, des adultes ont été exposés pendant 28 jours et par groupes de 5 (6 réplicats) à des concentrations comprises entre 133 et 1065 ng Sn/L de TBT. Les animaux ont été nourris *ad libitum*. Des informations ont été récoltées tout au long du test pour les variables biologiques suivantes : survie, taille de la coquille, poids sec du corps mou, nombre d'œufs cumulés produits par animal au cours du test. Remarquons que la température (22,2 ± 1,3°C *vs.* 19,5 ± 0,7°C) ainsi que la concentration en solvant (acétone, 20 *vs.* 2 µL/L) utilisées étaient légèrement différentes lors de ces deux tests. Enfin, des œufs de lymnées isolés de leur gangue gélatineuse ont été exposés au TBT durant 21 jours à des concentrations comprises entre 11 et 1995 ng Sn/L. Le taux d'éclosion moyen, la durée du développement embryonnaire et la taille à l'éclosion ont été déterminés.

Le modèle DEBkiss a tout d'abord été calibré en conditions témoins, sur la base des données de survie, de croissance et de reproduction issues des tests sur juvéniles et adultes. La différence de température entre les deux tests a été prise en compte à l'aide d'un facteur correcteur (température d'Arrhénius) appliqué à l'ensemble des constantes de flux énergétiques du modèle. Les valeurs de paramètres métaboliques ainsi calibrées ont ensuite été fixées afin d'estimer les valeurs des paramètres toxicologiques du modèle sur la base des données de survie, de croissance et de reproduction issues des tests sur juvéniles et adultes. Le mode d'action du xénobiotique le plus probable a été défini en comparant les prédictions du modèle obtenues en testant successivement les différents modes d'action possibles mentionnés au chapitre 3. Les données sur les performances embryonnaires n'ont pas été utilisées pour la calibration, mais pour vérifier les prédictions du modèle.

L'exposition au TBT a induit une faible diminution (< 10%) du taux d'éclosion des œufs exposés à 907 and 1995 ng Sn/L. Elle n'a pas entrainé d'augmentation significative de la fréquence de malformation des œufs, contrairement à ce qui a été observé au chapitre 2. Par ailleurs, le TBT a eu un impact négatif sur la survie des juvéniles, ainsi que sur la croissance et la reproduction des juvéniles et des adultes. Les effets ont été plus marqués chez les adultes que chez les juvéniles exposés aux mêmes concentrations nominales. Ce résultat n'est pas attendu dans le cadre de la théorie DEB, qui suppose que la sensibilité intrinsèque d'un animal à un toxique est constante au cours de son cycle de vie. Ce résultat peut s'expliquer par la différence de concentration en solvant entre les deux tests, qui a pu générer une différence de concentration réellement disponible pour les animaux. Cette différence a été prise en compte dans le modèle DEBkiss à l'aide d'un facteur correctif. Un facteur de saturation de l'absorption du TBT a par ailleurs été introduit afin de refléter

la toxicocinétique de façon fiable dans le modèle. Le modèle DEBkiss ainsi modifié a permis de décrire de façon adéquate les effets du TBT observés chez les juvéniles et les adultes. Les concentrations sans effet sur la survie et la croissance/reproduction ont été estimées à 49,5 [IC95% : 42,6 – 55,7] et 1386 [IC95% : 961 - 1910] ng Sn/L, respectivement. Ces valeurs coïncident avec les valeurs de NOEC disponibles dans la littérature. Le mode d'action suggéré par le modèle DEBkiss pour le TBT correspond à une diminution de l'énergie assimilée, liée soit à une moindre quantité de nourriture ingérée, soit à un plus faible rendement digestif. Cette hypothèse est corroborée par l'observation d'une diminution de la quantité de nourriture consommée par les animaux, diminution d'autant plus forte que la concentration en TBT est élevée. Pour ailleurs, nous avons observé un changement d'aspect de la glande digestive chez les animaux exposés : des dommages histologiques sur cette glande pourraient expliquer une moindre efficacité de la digestion des aliments chez les lymnées exposées au TBT. Cette diminution pourrait aussi s'expliquer par une action du TBT sur le système neuroendocrine de la lymnée (et en particulier sur les récepteurs aux rétinoïdes X) ayant des conséquences sur la production des peptides impliqués dans la nutrition et la croissance/reproduction de la lymnée. Les données acquises durant cette étude nous ont permis de progresser autour de la question du mode d'action du TBT chez la lymnée, mais sont encore insuffisantes pour conclure à ce sujet. Le modèle DEBkiss a été utilisé pour prédire les effets du TBT sur les embryons sous l'hypothèse d'une diminution de l'assimilation proportionnelle à la concentration d'exposition. Le modèle prévoit une diminution de la taille à l'éclosion et une augmentation de la durée embryonnaire, effets qui n'ont pas été observés expérimentalement.

En conclusion, ce chapitre démontre que le modèle DEBkiss permet de décrire de façon intégrative l'ensemble des effets du TBT au cours du cycle de vie de la lymnée. Des recherches complémentaires doivent être menées pour affiner les prédictions au stade embryonnaire, ce qui confirme les perspectives du chapitre 3. L'approche proposée n'a permis d'élucider complétement le mécanisme d'action du TBT chez cette espèce. Ceci s'explique par le fait que (i) les variables biologiques suivies au cours des tests de toxicité fournissent des informations non spécifiques du mode d'action du xénobiotique (*i.e.* la réduction d'assimilation de nourriture constatée peut être causée par plusieurs mécanismes agissant indépendamment ou de concert) et (ii) le modèle DEBkiss est générique en termes de mécanisme d'action. Une approche expérimentale et modélisatrice au niveau sub-individuel s'impose pour pouvoir déterminer si le TBT agit effectivement par perturbation endocrinienne ou non.

Chapitre 5 : Etude des effets des alkylphénols sur la lymnée

Ce chapitre décrit les résultats d'une exposition des embryons, juvéniles et adultes de lymnée à différents alkylphénols (4-nonylphénol, 4-n-nonylphénol, and 4-tert-octylphénol) ayant une action œstrogénique chez les vertébrés. A ce titre, ces composés font actuellement l'objet d'intenses recherches ayant une finalité en évaluation de risque. Les résultats de ces études publiés dans la littérature ne sont pas cohérents. Chez les mollusques prosobranches, ces xénobiotiques peuvent induire une augmentation de la fécondité, généralement associée à une perturbation endocrinienne, mais cela n'est pas observé chez toutes les espèces de mollusques. Nous souhaitons donc voir si de tels effets surviennent aussi chez la lymnée.

Pour ce faire, nous avons réalisé des expositions d'embryons, de juvéniles et d'adultes de lymnée à ces alkylphénols. Un composé œstrogénique de synthèse, le 17α-ethinylœstradiol (EE2), a été utilisé comme témoin position pour ces tests de toxicité. Un test de 28 jours et un test de 56 jours ont été réalisés sur des juvéniles suivant les protocoles utilisés au chapitre 2. Un test de 35 jours sur les adultes et un test de 21 jours sur embryons ont été réalisés suivant les protocoles utilisés au chapitre 3. Le tableau 5.3 p 62 du manuscrit présente la liste des variables biologiques ayant été mesurés lors des tests : nous avons choisi d'étudier les effets individuels de façon la plus exhaustive possible, ce qui nous a conduit à considérer 19 variables biologiques différentes lors de nos tests de toxicité. Les données issues de ces tests ont été analysées selon une approche de statistique

descriptive classique en écotoxicologie, similaire à celle présentée dans le chapitre 2. Chaque xénobiotique a été étudié dans une très large gamme de concentration, comprise entre 0,05 et 1000 µg/L de produit pur. Les concentrations en EE2 ont été mesurées dans l'eau d'exposition et dans les organismes. Les concentrations réelles en alkylphénols n'ont pas pu être déterminées au cours de la thèse.

Les résultats de ces différents tests de toxicité ont été cohérents, même si le plan expérimental variait (en termes de durée et de nombre d'animaux étudiés) entre ces tests. Le tableau 5.5 p 65 du manuscrit résume ces résultats. Les composés testés n'ont pas induit d'effet sur les animaux exposés à des concentrations environnementales en xénobiotiques. Des effets toxiques sont apparus aux plus fortes concentrations nominales testées. Ainsi, l'EE2, le 4-nonylphénol et le 4-tert-octylphénol ont induit une diminution de la survie à 50 µg/L et 1000 µg/L, respectivement. La fécondité des animaux exposés à 500 et 1000 µg/L de 4-nonylphenol et de 4-tert-octylphénol a été significativement réduite. L'absence d'effets de l'EE2 et des alkylphénols testés aux concentrations environnementales confirme les résultats publiés par d'autres auteurs pour la lymnée. L'innocuité de ces composés chez la lymnée peut s'expliquer par (i) leurs propriétés toxico-cinétiques *e.g.* biotransformation et élimination des alkylphénols, ou régulation homéostatique de la concentration en EE2 du fait de sa similarité avec des hormones naturellement présentes chez la lymnée ou (ii) de leurs propriétés toxico-dynamiques *e.g.* faible affinité des xénobiotiques pour les récepteurs à l'EE2 ou aux composés œstrogéno-mimétiques tels que les alkylphénols chez la lymnée. Par ailleurs, l'absence de tels récepteurs, ou le fait que ces récepteurs (s'ils existent) soient impliqués dans des fonctions physiologiques indépendantes de la croissance et de la reproduction, pourraient expliquer les résultats obtenus chez la lymnée. Ces résultats suggèrent en outre qu'il est déconseillé d'utiliser l'EE2 comme témoin positif de perturbation endocrinienne chez la lymnée, puisque ce composé n'induit pas d'effets chez les animaux exposés aux concentrations pertinentes.

Chapitre 6 : Discussion

La discussion de ce travail de thèse aborde successivement quatre points. Premièrement, une synthèse de nos réflexions/hypothèses relatives aux mécanismes de perturbation endocrinienne chez la lymnée est conduite, en comparaison avec les autres mollusques. L'exposition des lymnées à des hormones stéroïdiennes synthétiques proches de celles des vertébrés (17α-méthyltestostérone - MT - et EE2) à des concentrations égales ou supérieures aux concentrations environnementales n'a pas induit d'effets délétères chez les animaux exposés, et ce à aucun stade du développement. Cette absence d'effet de la MT peut s'expliquer par des régulations homéostatiques de la concentration et/ou par l'absence de récepteur aux androgènes chez la lymnée. L'analyse des concentrations internes suggère que l'EE2 peut s'accumuler dans les tissus de la lymnée jusqu'à des concentrations très élevées sans entrainer d'effet délétère. La présence du récepteur aux œstrogènes est suspectée chez *L. stagnalis* mais reste à démontrer. Les résultats obtenus durant cette thèse n'ont pas permis de démontrer que la testostérone et l'œstradiol, caractéristiques des systèmes endocrines vertébrés et naturellement présents chez la lymnée, sont effectivement impliqués dans le control endocrinien du développement, de la croissance ou de la reproduction chez ce gastéropode. L'exposition des lymnées à des alkylphénols (composés mimétiques ou antagonistes des œstrogènes chez les vertébrés) n'a pas induit d'effets chez la lymnée. Cependant, nos travaux de thèse ainsi que les récents résultats de la littérature montrent que certains composés mimétiques ou antagonistes des androgènes chez les vertébrés peuvent induire des effets sur la survie, le développement, la croissance et/ou la reproduction des lymnées. Etant donné que le récepteur aux androgènes est absent chez la lymnée, les mécanismes d'effets de ces molécules sont probablement différents des mécanismes proposés pour expliquer les effets de ces produits chez les vertébrés. Cette question a été approfondie en utilisant le TBT comme molécule modèle. L'une des explications fréquemment apportée dans la littérature pour expliquer les effets du TBT chez les mollusques prosobranches est l'augmentation du niveau de testostérone libre. Cette explication ne permet pas d'expliquer les

effets observés à des faibles concentrations en TBT chez *L. stagnalis*, car cet organisme est capable de réguler la concentration en testostérone libre dans une certaine mesure, et ce par estérification. La seconde hypothèse issue de la recherche sur les prosobranches consiste en une interaction entre le TBT et le récepteur aux rétinoïdes X (RXR). Ces récepteus étant présent chez la lymnée, et notamment observé dans les tissus impliqués dans le control neuroendocrinien de la croissance (*e.g.* light green cells), cette hypothèse est vraisemblable chez *L. stagnalis*. Suite à cette thèse, un projet de recherche visant à caractériser la structure du RXR et l'affinité de différents xénobiotiques présentant une activité endocrinienne chez les vertébrés à ce récepteur à récemment démarré.

Deuxièmement, les avantages et les limitations du modèle DEBkiss pour l'évaluation du risque des produits chimiques en général, et plus particulièrement des perturbateurs endocriniens, sont présentés. Les principaux avantages des modèles issus de la théorie DEB pour l'analyse des données d'écotoxicité sont les suivants :

- ces modèles permettent l'analyse des données au-delà de la durée d'exposition et des concentrations testées ; il n'est pas nécessaire de maintenir la concentration constante au cours du test.
- l'ensemble des données formant la courbe concentration – réponse est utilisé pour l'analyse.
- l'ensemble des effets observés au long du cycle de vie peuvent être intégrés en un seul et même modèle.
- une fois calibrés, ces modèles permettent de prédire les réponses des animaux à des scénarios d'exposition différents de ceux qui ont été étudiés expérimentalement.

Le modèle DEBkiss constitue une simplification du modèle DEB standard qui facilite le traitement des données d'écotoxicité chez les petits invertébrés. Ce modèle étant plus simple, il est plus facile à calibrer à partir des données de bioessais classiquement disponibles. Il permet de combiner des jeux de données issues de test de toxicité conduits dans des conditions différentes en termes de température, solvant, niveau de nourriture, nombre d'individus testés, etc. Ceci permet une utilisation optimale des données disponibles pour le chercheur. Cependant les données issues des protocoles standardisés ne suffisent pas à calibrer le modèle : cette limitation ne favorise pas l'utilisation de ce modèle dans un contexte réglementaire. L'analyse des données des bioessais conduits avec des molécules suspectées d'agir en tant que perturbateur endocrinien chez les vertébrés et les mollusques prosobranches, telles que le TBT, n'a pas nécessité d'adaptation particulière du modèle DEBkiss en termes de toxico-dynamique : les mécanismes d'action génériques à l'ensemble des xénobiotiques se sont avérés suffisant pour décrire les effets du TBT chez *L. stagnalis*. Cependant, comme la thèse n'a pas permis de démontrer que le TBT agit effectivement comme un perturbateur endocrinien chez *L. stagnalis*, nous ne pouvons pas conclure quant à la pertinence du modèle DEBkiss sous sa forme actuelle pour l'étude de la perturbation endocrinienne.

Troisièmement, nous proposons des recommandations relatives au plan d'expérience et au choix des variables biologiques en fonction contexte (réglementaire *vs.* recherche) et du type d'analyse de données utilisé (statistiques *vs.* modélisation mécaniste). Dans un contexte réglementaire, les protocoles standardisés (*e.g.* par l'OCDE) doivent être utilisés. Le développement de ces protocoles standardisés étant actuellement en cours pour la lymnée, nous recommandons de tenir compte des observations suivantes :

- la croissance des juvéniles et le développement embryonnaire peuvent se révéler des critères d'effets sensible aux toxiques et en particulier aux perturbateurs endocriniens. L'étude de ces variables est potentiellement utile pour l'extrapolation des effets au niveau des populations.
- le poids sec est un estimateur plus faible que la taille de la coquille lors de l'étude des effets des xénobiotiques sur la croissance de la lymnée.
- le comptage du nombre d'œufs produit par individu est l'étape la plus longue du test de reproduction avec la lymnée. Afin d'alléger le test, le poids sec des pontes peut être utilisé comme proxy pour la mesure de fécondité.

- une durée de test de 21 à 35 jours est probablement suffisante pour détecter les effets des xénobiotiques étudiés sur la croissance des juvéniles, la fécondité des adultes et le développement embryonnaire.
- l'utilisation de solvants doit être évitée si possible. Néanmoins l'ajout de 20 µL/L d'acétone n'a pas d'effet significatif sur les juvéniles et les adultes de lymnée.

Les recommandations sont les suivantes lorsque les données du test de toxicité sont analysées grâce au modèle DEBkiss :

- il est nécessaire de collecter des valeurs de survie, de poids et de fécondité en continu au cours du test de toxicité, et ce sur une partie suffisamment longue du cycle de vie (comprenant les stades juvéniles et adulte).
- certaines données complémentaires (*e.g.* durée du développement embryonnaire et poids des nouveau-nés) peuvent être utiles pour déterminer le mécanisme d'action du toxique.
- le nombre d'individus étudié par concentration peut être plus faible que lors de bioessais standards et peuvent varier d'une concentration à l'autre. Cependant, le nombre d'animaux témoins doit être plus élevé que le nombre d'animaux exposés au xénobiotique pour permettre une bonne calibration du modèle. Utiliser seulement quatre ou cinq animaux par modalité de traitement semble insuffisant au vu de la grande variabilité des traits de vie et des réponses chez la lymnée : le nombre optimal d'individu requis pour une interprétation fiable des effets reste à déterminer.

Quatrièmement, nous présentons deux perspectives de recherches majeures liées à notre travail de thèse. Dans ce travail de thèse, nous avons raisonné sur les performances moyennes d'un lot d'individus exposés aux xénobiotiques. Etant donné que la variabilité de traits de vie et de réponse aux xénobiotiques est très importante chez la lymnée, cette variabilité devrait être prise en compte lors de l'analyse des effets grâce aux modèles DEBkiss. Il est possible de modéliser les performances de chacun des individus présents dans le test de toxicité, mais cette analyse est lourde en termes de puissance de calcul et de temps. Par ailleurs, nous avons raisonné ici à l'échelle de l'individu entier. Or, selon la définition réglementaire, l'étude des effets des perturbateurs endocrinien nécessite de prédire la cascade d'évènements ayant lieu depuis l'échelle moléculaire jusqu'à la population. Dans ce contexte, il serait souhaitable de relier les informations dont nous disposons chez la lymnée au niveau moléculaire avec les informations qui peuvent être tirées de l'analyse des données de tests de toxicités par le modèle DEBkiss. Un lien qualitatif pourrait être établit au travers d'un AOP, tandis que le modèle DEBkiss, après avoir été modifié afin de prendre en compte des effets à l'échelle sub-individuelles, apporterait des informations quantitatives utiles.

Sažetak

Endokrini disruptori (ED) su hemikalije poreklom iz spoljašnje sredine koje izazivaju štetne posledice po organizam dejstvujući na endokrini sistem. Ove hemikalije su sastojci mnogih produkata svakodnevne upotrebe, kao što su plastične flaše, hrana, deterdženti, kozmetika, a takođe su i komponente mnogih pesticida i biocida. Kada se nađu u organizmu, ED prvenstveno utiču na razvoj, rast i razmnožavanje tako što narušavaju ravnotežu endokrinog sistema. Ove biološke odlike su ključne za održanje populacije, što je ujedno i cilj kojem se teži u zaštiti životne sredine kada su u pitanju vodeni beskičmenjaci. Ograničeno znanje o endokrinologiji beskičmenjaka otežava identifikaciju i procenu efekata ED na nivou jedinke. Između ostalog, slaba prihvaćenost mehanističkih modela[1] u ekotoksikološkim istraživanjima i zaštiti životne sredine onemogućava predviđanja efekata koje ED ispoljavaju na populacije beskičmenjaka. Prema tome, ova doktorska teza ima za cilj unapređenje metoda za testove toksičnosti i unapređenje načina analize podataka za procenu i predviđanje efekata ED na velikog barskog puža *Lymnaea stagnalis*. Ovaj vodeni puž je osetljiv na raznovrsne hemikalije, uključujući i ED, i stoga je on izabran kao test vrsta u ovoj doktorskoj tezi. Ova vrsta takođe ima velikog značaja u tekućem procesu standardizacije testova razmnožavanja mekušaca koji predvodi međunarodna Organizacija za ekonomski razvoj i saradnju (OECD).

U ovoj tezi ispitivana je osetljivost velikog barskog puža na androgene i estrogene hemikalije, to jest jedinjenja koja utiču na funkcije organizma od značaja za razvoj i održanje muških i ženskih karakteristika. Izloženost velikog barskog puža steroidnim hormonima bi moglo prouzrokovati androgene ili estrogene efekte, slične onima koji se viđaju kod kičmenjaka i još nekih vrsta vodenih puževa. Opažanjem uticaja steroidnih hormona na razvoj bioloških osobina velikog barskog puža bi moglo podržati hipotezu da steroidni hormoni imaju ulogu u ostvarivanju tih uticaja putem endokrinog sistema. Dalje, ako izlaganje velikog barskog puža drugim androgenim hemikalijama, npr. organokalajnim jedinjenjima, ili estrogenim hemikalijama, npr. alkilfenolima, rezultira efektima sličnim onima izazvanim steroidima, to bi predstavljalo uticaj na endokrini sistem. Da bi ove hipoteze bile testirane, niz testova toksičnosti je izveden, a dobijeni podaci su bili analizirani standarnim statističkim metodama kao i mehanističkim modeliranjem toksikokinetičkih i toksikodinamičkih (TKTD) procesa.

Rezultati testova toksičnosti na sintetičke steroidne hormone, 17α-metiltestosteron (MT, androgen) i 17α-etinilestradiol (EE2, estrogen), su pokazali nepostojanje efekata ovih hemikalija na razne test parametre vezane za razvoj, rast i razmnožavanje velikog barskog puža pri koncentracijama koje se mogu naći u životnoj sredini. Rezultati testova dalje ukazuju na verovatnoću da ovi steroidi nisu uključeni u signalne puteve hormona koji definišu posmatrane biološke karakteristike puža. Alkilfenoli takođe nisu prouzrokovali efekte kada su puževi bili izloženi koncentracijama koje se mogu naći u životnoj sredini. Ovo je objašnjeno brzim metabolizmom i efikasnim uklanjanjem alkilfenola iz tela. Pošto MT i EE2 verovatno ne izazivaju efekte kod velikog barskog puža karakteristične za steroidne hormone, zaključeno je da ove dve hemikalije ne bi trebalo da služe kao pozitivna kontrola u testiranjima toksičnosti ED kada je u pitanju ova vrsta puža.

Testovi toksičnosti organokalajnih jedinjenja tributilkalaj (TBT) i trifenilkalaj (TPT) su pokazali da je veliki barski puž osetljiv na niske koncentracije ovih hemikalija koje se smatraju za ED. Uprkos sličnosti između ova dva jedinjenja u pogledu njihovih fizičko-hemijskih i strukturnih osobina, efekti TBT i TPT su se međusobno razlikovali. U testovima toksičnosti sa pre-adultnim i adultinim puževima, test parametri koji nisu vezani za preživljavanje su bili mnogo više osetljivi na TBT nego na TPT. Kada su jaja, poreklom od puževa koji nisu bili izloženi toksikantima, bila

[1] Mehanistički model u ovom slučaju predstavlja skup matematičkih jednačina i pravila kojima se objašnjavaju procesi u sistemu, oslanjajući se na mehanizme koji su ogovorni za te procese.

ekstrahovana iz njihovih želatinoznih vrećica i izložena direktno organokalajnim hemikalijama, stepen toksičnosti je bio obrnut u poređenju sa pre-adultima i adultima (TPT je izazvao mnogo jače efekte). Razlog za razliku u efektima izazvanim sa TBT i TPT i dalje ostaje nejasan; bilo je nagađano da su u pitanju razlika u alkilaciji ova dva jedinjenja i/ili razlike u ekspresiji pojedinih receptora. Što se tiče osetljivosti testova, najosetljiviji parametar za TBT je bio učestalost poliembrionije jaja kod puževa koji su bili izloženi toksikantu, dok je za TPT to bilo vreme do izleganja kod jaja koja su bila direktno izložena ovom jedinjenju.

Da bi predstavili pogodnosti koje TKTD analiza pruža u izučavanju efekata ED na velikom barskom pužu, prvo smo morali da osmislimo najpodesniji test dizajn. U studiji slučaja u kojoj je veliki barski puž bio izložen acetonu, podesnost različitih setova podataka za kalibraciju TKTD modela je bila ocenjivana. Eksperimenti nad velikim barskim pužem su bili izvođeni; embrioni, pre-adulti i adulti su bili izloženi različitim koncentracijama acetona. Test parametri kao što su preživljavanje i oni koji su vezani za razvoj, rast i razmnožavanje su bili praćeni tokom vremena. Dobijeni setovi eksperimentalnih podataka su bili analizirani pomoću "DEBkiss" modela – modela koji spada u kategoriju TKTD modela i koji je baziran na teoriji dinamičkog energetskog budžeta (DEB, Dynamic Energy Budget theory). Kada se radi o kalibraciji DEBkiss modela, upotpunjenost setova podataka je pre uticala na preciznost nego na tačnost parametara DEBkiss modela. Na osnovu toga što je kalibracija DEBkiss modela sa potpuno različitim setovima podataka rezultirala u dobijanju veoma sličnih vrednosti parametara modela, može se zaključiti da su vrednosti DEBkiss parametara odlično procenjene. Podaci o oporavku puževa nakon izloženosti acetonu su doprineli iznenađujuće malo poboljšanju preciznosti kojom su parametri bili procenjeni. Dalje, model koji je kalibrisan sa najpotpunijim setom podataka u okviru jedne uzrasne strukture najbolje je predvideo najnepotpuniji set podataka u okviru iste uzrasne strukture. Štaviše, embrionalni razvoj i razvoj, rast i razmnožavanje pre-adultnih i adultnih puževa su se mogli predvideti birajući jedan isti način toksičnog delovanja – smanjenje efikasnosti asimilacije. Ova studija je istakla izuzetnu važnost podataka o embrionalnom razvoju za odabir odgovarajućeg načina delovanja toksikanata.

DEBkiss je potom bio primenjen za analizu podataka dobijenih iz dva potpuno nezavisna testa toksičnosti u kojima su pre-adulti i adulti bili izloženi različitim koncentracijama TBT. DEBkiss model je jedino bio modifikovan u smislu fleksibilnosti pri različitim temperaturrama i prilagođenosti za razlike u biodostupnosti TBT usled različitih koncentracija rastvarača između dva testa. TBT je negativno uticao na preživljavanje, rast i razmnožavanje puževa. Analiza podataka nije zahtevala dodatne hipoteze o načinu dejstva TBT. Preciznije, efekti TBT su bili odlično objašnjeni načinom toksičnog delovanja koji je uobičajen za hemikalije koje nisu ED – smanjenjem stope unosa hrane. Smanjenje unosa hrane dovodi do toga da je organizmu dostupno manje energije nego što je potrebno, što vodi do smanjenja stope rasta i smanjenja maksimalne veličine jedinke. Smanjen unos hrane i manja veličina tela uzrokuje smanjenje stope razmnožavanja. Stoga, TBT je uticao na smanjenje razmnožavanje indirektno.

Na kraju, date su preporuke kako dizajnirati testove predviđene za TKTD analizu kao i kako unaprediti protokole za tekuću standardizaciju testova razmnožavanja velikog barskog puža. Najveća prednost TKTD analize u vezi sa testovima toksičnosti je fleksibilnost u njihovom dizajniranju. Da bi se efekti toksikanata objasnili na najbolji način uz pomoć TKTD modela, neophodno je da se rast i razmnožavanje individua prati u okviru jedne iste grupe životinja tokom većeg dela životnog ciklusa, npr. počevši od juvenilnog stadijuma pa sve do duboko u zrelo doba. Što se tiče razvoja test protokola od strane OECD, preporučeno je da se u buduće testove uključe i osmatranja kvaliteta jaja kao i embrionalnog razvoja, pošto to može povećati osetljivost i značaj testova toksičnosti. Bilo bi takođe veoma korisno da se u standardne testove uvede merenje veličine puževa kao dodatni parametar. Hemikalije mogu uticati na ovaj parametar direktno, što vodi do indirektnog efekta na razmnožavanje, a samim tim do posledica na dinamiku populacije.

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